Scent marking in mustelids and banks voles.

Analyses of chemical compounds and their behavioural significance.

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SCENT MARKING IN MUSTELIDS AND BANK VOLES

ANALYSES OF CHEMICAL COMPOUNDS AND

THEIR BEHAVIOURAL SIGNIFICANCE

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ABSTRACT

Scent communication has been investigated in anal sac secretion in mustelids and in preputial gland secretion in bank voles. In mustelids compound and compound combinations were related to phylogenetic groups among a selection of taxa. Some of the compounds were species-specific and the relative amounts of mutual compounds varied between the species.

Variations occurred in the generalized pattern characteristic of the species. These have been analyzed in a stoat population.

In stoats the variation in the lower molecular weight range was persistent. At a comparison between anal sac secretion and skin gland secretion no compounds, identified from the anal sac, could be refound in skin gland secretion. These results are in agreement with the fact that the respective secretions act at two different types of behaviour, viz. anal drag marking and body rubbing.

The investigation of the individual variation of the anal sac secretion was extended by an examination of 10 individuals of mink for 11 months. The individual pattern persistent during the period relied on a complex of compounds in the higher molecular weight range.

In bank voles n-hexadecyl acetate is present in the preputial secretion and is deposited in the marking urine, but not in the metabolic urine. The acetate is important for dominance interactions.

Subordinate male bank voles, treated with testosterone, showed an increased amount of hexadecyl acetate in preputial gland secretion.

UNIV. LEIDEN BIBL.

LIST OF PAPERS

The thesis is based on the following papers:

I Brinck, C., Erlinge, S. and Sandell, M. (in press). Anal sac secretion in mustelids - A comparison. - J. Chem. Ecol.

II Brinck, C., Gerell, R. and Odham, G. 1978. Anal pouch secretion in mink Mustela vison. - Oikos 30: 68-75.

III Brinck, C. Classification of individuals in a population of Mustela erminea by GC/MS. - Manuscript.

IV Erlinge, S., Sandell, M. and Brinck, C. 1982. Scent-marking and its territorial significance in stoats, Mustela erminea. - Anim. Behav. 30: 811-818.

V Brinck, C. and Hoffmeyer, I. Marking urine and preputial gland secretion of male bank voles (Clethrionomys glareolus L.): Chemical analyses and behavioural tests. - Manuscript.

VI Brinck, C., Hoffmeyer, I. and Westlin, L. Effect of testosterone on the structure and secretion of the preputial glands in subordinate male bank voles, (Clethrionomys glareolus L.). - Manuscript.

FÖRORD

Att producera en doktorsavhandling är ofta ett grupparbete. Inte minst är det fallet när arbetsfältet ligger mellan två akademiska ämnesgrenar och därmed kommer att omfatta element ur bådadera. Det gäller sålunda den kemiska ekologin, vars metodik och arbetsprinciper hämtas från kemi och fysik, medan material och tillämpningar i fält och på laboratorium berör olika delar av biologin.

I min utbildning har jag haft förmånen och glädjen att ha stimulerande och skickliga lärare som professorna Stina och Einar Stenhagen, Göteborgs universitet, och Gösta Ehrensvärd, Lunds universitet. Huvuddelen av mitt arbete har under senare år varit förlagt till Ekologihuset i Lund, vars rika resurser och skickliga personal kommit att spela en stor roll för mitt arbete. Jag vill här särskilt tacka docent Göran Odham, föreståndare för avdelningen för ekologisk kemi, och docent Sam Erlinge, ledare för vertebratekologiska forskningsgruppen samt docent Jan Löfqvist, som med sin breda sakkunskap bistått med värdefulla synpunkter. Ett varmt tack går till ekokemiska avdelningen, särskilt Gunilla Westerdahl. Oumbärliga vid avhandlingens produktion har varit Steffi Douwes, Gunilla Lindquist och Anne Odham, som var och en inom sitt område gjort en viktig insats.

SUMMARY

Scent communication plays an important role in animal behaviour. This thesis concerns scent compounds interpreted in relation to the behaviour of mustelids and small rodents.

The following aspects have been considered: The abundant occurrence of biologically active scent substances in secretion systems, and the diverse biological role of compounds involved.

In mustelids, an important source of the scent components are the anal glands at the base of the tail (Fig. 1). The glandular secretion has a pungent odour - some humans regard it as disgusting - due to sulphur compounds of high volatility. The human nose can discriminate between secretion from the different mustelids, e.g. the stoat, the weasel and the mink.

MUSTELID STUDIES

Paper I deals with compounds and compound combinations related to extant phylogenetic groups among a selection of taxa. The anal sac secretion from the species was analyzed with GC. The gas chromatograms of the compounds can be regarded as "fingerprints" - reflecting the identity of the species. The chromatograms were very similar in the low molecular weight region between species belonging to the genus Mustela; viz. M. erminea (stoat), M. nivalis (weasel), M. vison (mink) and M. putorius (polecat), whereas Martes martes (marten), Lutra lutra (otter) and Meles meles (badger) each showed a divergent pattern. Benzaldehyde was the predominant compound in the secretion of the marten. Sulphur-containing compounds were characteristic of the species of Mustela.

Some of the compounds were species-specific and the relative amounts of mutual compounds varied between the species. This makes exchange of information possible between co-existing mustelids. Some compounds may contribute to the defence behaviour.

The investigation of the individual variation of the anal sac secretion was extended by an examination of 10 individuals of mink for 11 months (Paper II). The individual pattern persistent during the period relied on a complex of compounds in the higher molecular weight range. No sex differences were found. Probably such compounds form a pattern, serving at the recognition of the individuals, while more volatile substances signal changes or

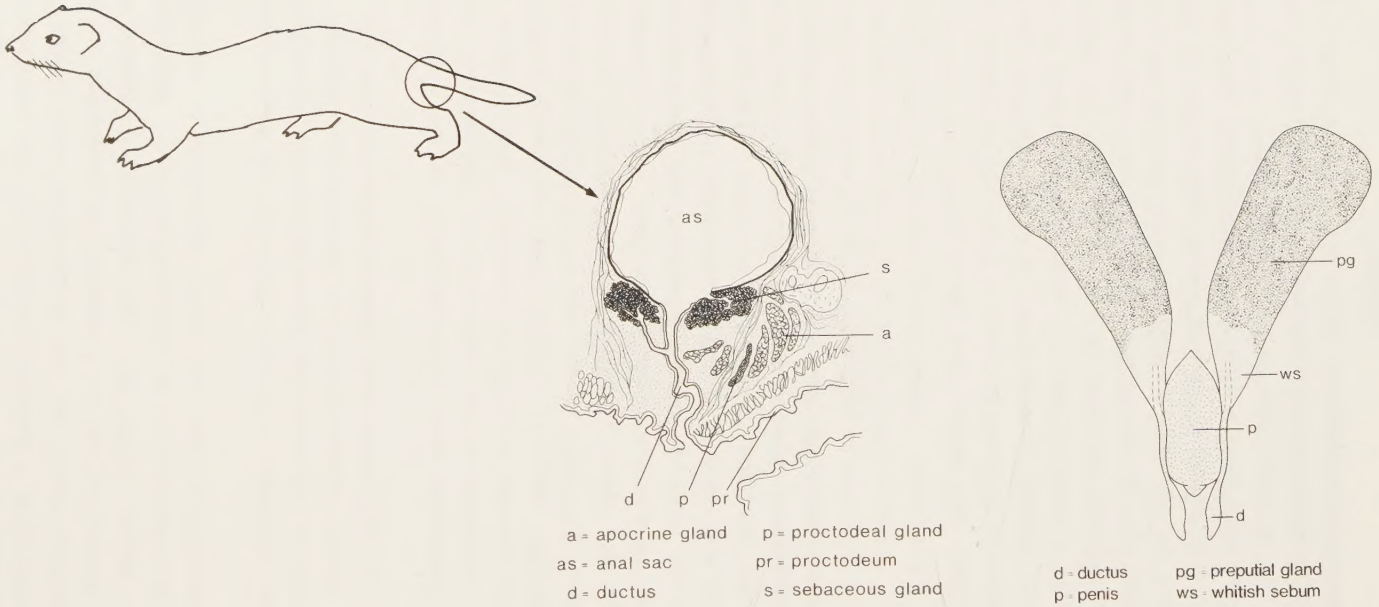

Fig. 1A. Anal gland in a stoat (Mustela erminea).

1B. Preputial glands of a dominant male bank vole (Clethrionomys glareolus).

differences in activity or status. Thus, the animals may utilize different parts of their scent spectrum for different purposes.

Individual variations occur within the generalized pattern characteristic of the species. In Paper III quantitative variations of selected substances have been analyzed in a stoat population. While the females generally produced less of the compounds, there were large sexual variations in the relation between the compounds. The variation could not be related to differences in sex, dominance or activity. Various steps of the biosynthesis of the compounds, after the glands had been emptied, might be involved.

In Paper IV the variations of the secretion of selected male stoats were analyzed on two occasions during a short period of time. For a number of compounds, the pattern of variation between the individuals was persistent. At a comparison between anal sac secretion and skin gland secretion no compounds, identified from the anal sac, could be refound in skin gland secretion. These results are in agreement with the fact that the respective secretions act at two different types of behaviour, viz. anal drag marking and body rubbing. Different messages are probably transmitted by the two ways of marking; body rubbing occurs associated with agonistic interactions and seems to have threat significance. Anal drag is probably used to impregnaten an area with the individual's odour. Scent-marking is considered to be important in allowing assessment of the asymmetry in a conflict, as between a territory owner and an intruder.

The results form a basis for the studies on the two different purposes of the compounds: 1) the recognition of the species or species groups, at the same time a potent instrument for their separation during the evolution and 2) the communication informing about activities and status and visualized as acts of behaviour.

The material reveals that certain substances represented among all the taxa investigated, probably have had a great constancy during the evolution, while others characterize species or species

groups. The former may belong to the individual´s life-long chemical pattern of recognition, while the latter may be part of the signal substances, informing about activities or status.

RODENT STUDIES

In Paper V it is shown that in bank voles the preputial gland secretion is connected with urinary marking. N-hexadecyl acetate is present in the preputial secretion and is deposited in the marking urine, but not in the metabolic urine. Both males and females react strongly to urine mixed with the secretion when the mixture is presented as an alternative to metabolic urine. The acetate is an important compounds in the secretion which is essential in marking pattern and dominance interactions.

Paper VI. Subordinate male bank voles were treated with testosterone in order to determine its effect on their preputial gland secretion. The hormone caused a clear hypertrophy of the glands, thus indicating an increased activity of the glands. There was a striking increase in the number of granules and lipid droplets in the treated specimens. The hormone treatment was followed by an increased amount of hexadecyl acetate in preputial gland secretion.

ANAL SAC SECRETION IN MUSTELIDS - A COMPARISON

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ABSTRACT

The chemical compositions of anal sac secretions of seven mustelid species were examined by thin layer chromatography, gas chromatography, and mass spectrometry. The analyses showed great similarities between species belonging to the genus Mustela, i.e., M.erminea, M.nivalis, M.vison, and M.putorius, whereas Martes martes, Lutra lutra, and Meles meles each showed a different pattern. Benzaldehyde was the predominant compound in the secretion of M.martes. Sulphur-containing compounds (thietanes and dithiacyclopentanes) were characteristic for the Mustela species. Some of the compounds were species--specific and the relative amounts of the compounds in common varied between the species. The chemical results are in agreement with systematics at the generic level. Species-specific chemical composition makes exchange of information possible between co-existing mustelids. The presence of the sulphur--containing compounds in the small mustelids may be an effect of the defence function of the anal sac secretion.

INTRODUCTION

The mustelids are small to medium-sized carnivores which have diversified to exploit different habitats and food resources (Ewer 1973). They are typically solitary and the individuals in a population are usually distributed according to a territorial pattern (Powell 1979). Often two or more species occur in the same area which may result in interference (Erlinge 1972, King and Moors 1979, Simms 1979).

Scent communication is important in both intra- and probably also in interspecific interactions. The scent material is deposited at certain sites according to specific behaviours (Ewer 1968, Macdonald 1980). Besides urination and defaecation, body rubbing and anal drag are characteristic scent marking behaviours in mustelids (Goethe 1964, Erlinge et al. 1982).

The microscopic anatomy of anal sacs in some mustelids was described by Stubbe (1970). Four types were distinguished, i.e., the Meles-, Lutra-, Martes-, and Mustela-type. The anal sacs of Martes and Mustela represented a more advanced type with a high concentration of tubular and alveolar glands.

The chemical compositions of the anal sac secretion have been examined in some mustelids, i.e., mink (Mustela vison, Schreber) by Sokolov et al. (1975, 1980), Schildknecht et al. (1976), and Brinck et al (1978), otter (Lutra lutra L.) by Gorman et al. (1978), stoat (Mustela erminea L.) and ferret (Mustela putorius furo L., a domestic form of uncertain origin) by Crump (1978, 1980a, 1980b), and badger (Meles meles L.) by Brundin et al. (unpubl.).

On the basis of published information and our analyses, we compared the chemical composition of the anal sac secretions in eight mustelid species. The aim was to examine intergeneric and interspecific differences and similarities, and to make comparisons with phylogenetic relationships.

MATERIALS AND METHODS

ANIMALS

Anal sac secretions were obtained from 12 anaesthetized Meles meles L. (8 ♂ and 4 ♀), 5 anaesthetized Lutra lutra L. (1 ♂ and 4 ♀), 14 killed Martes martes L. (9 ♂ and 5 ♀), 47 killed and 3 anaesthetized Mustela erminea L. (34 ♂ and 16 ♀), 39 killed and 3 anaesthetized Mustela nivalis L. (23 ♂ and 19 ♀) and 15 anaesthetized Mustela putorius L. (8 ♂ and 7 ♀). Some complementary studies were performed on Mustela vison (3 ♂). All animals were caught in southern Sweden except one specimen of Lutra lutra which was captured in middle Norway.

SAMPLING TECHNIQUE

From anaesthetized Meles meles and Mustela putorius and killed M.vison, the secretion was collected by means of a catheter (Brinck et al. 1978). Before collecting the secretion of killed or anaesthetized individuals of the other species (i.e., Lutra lutra, Martes martes, Mustela erminea, and M.nivalis) the area around the openings of the anal sacs was washed with diethyl ether. The contents were then pressed out from the sacs and absorbed on cotton plugs which had previously been washed with ethanol and diethyl ether. The organic material was extracted with 0.5 ml methylene chloride. The samples were stored at -20°C until examined. The secretion of each individual was analyzed separately.

THIN LAYER CHROMATOGRAPHY (TLC)

Analytical TLC was run on commercial silica gel plates (E. Merck, Darmstadt, West Germany, Kieselgel 60F Thickness 0.25 mm) with light petroleum b.p. 60-80°C-diethyl ether 90:10 (v/v) as solvent. The spots were visualized by exposure to iodine vapours and also

by spraying with sulphuric acid (5 % solution in ethanol) followed by charring at 180°C.

GAS CHROMATOGRAPHY (GC)

Analytical GC was performed on Perkin Elmer Model 900 and 3920 instruments equipped with FID. A 25 m glass capillary column (i.d. 0.3 mm) dynamically coated with OV-101 in the laboratory was used for the secretions from *Mustela erminea*, *M.nivalis*, *M. putorius* and *M.vison*. The carrier gas flow was 1.4 ml min^{-1}. Make-up gas corresponding to 25 ml min^{-1} was employed. For the low molecular weight components a split giving a ratio of 1:30 was used. For the secretions from *Martes martes*, *Lutra lutra* and *Meles meles* a 25 m fused silica capillary column (i.d. 0.2 mm) dynamically coated with OV-101 was used. The split was closed for 1 min after injection. Program 4°C min^{-1} from ambient -230°C.

MASS SPECTROMETRY (MS)

Mass spectra were obtained with a Varian MAT 112 GC/MS instrumentation. In this GC/MS combination, gas chromatograph was a Varian model 1400 equipped with a 25 m fused silica capillary column. The helium carrier gas flow was 2 ml min^{-1}. The split ratio at the injection port was 1:30. The spectra of *cis*- and *trans*-2,4-dimethylthietanes were obtained from a Ribermag 10-10 computerized GC/MS system. The mass spectra were compared with spectra from synthetic products prepared in the laboratory or with spectra available in literature (Table 1). The synthetic thietanes used were prepared by reacting different dioxan-2-ones with potassiumthiocyanate.

INFRARED SPECTROMETRY (IR)

Infrared spectrometry (IR) was performed on a Perkin Elmer Model 298.

TABLE 1

Reference substances used for identification of compounds in anal sac secretions in the examined Mustelids.

	Synthetized in the laboratory*	Commercially available	Literature
2,2-dimethylthietane	x		
2,4-dimethylthietane (cis- and trans-)	x		
2,3-dimethylthietane (cis- and trans-)	x		
3,3-dimethylthietane	x		
2-ethylthietane	x		
3-ethylthietane	x		
indole		x	
p-aminoacetophenone		x	
benzaldehyde		x	
2-propylthietane			(Crump 1980a)
3,3-dimethyl-1,2-dithiacyclopentane			(Sokolov et al. 1980)
3-ethyl-1,2-dithiacyclopentane			(Crump 1980a)
2-pentylthietane			(Crump 1980a)
3-propyl-1,2-dithiacyclopentane			(Crump 1980a)

*The syntheses were performed by A. Brundin, Inst. Organic Chemistry, Univ. of Umeå, Sweden (unpubl.).

RESULTS AND DISCUSSION

COMPOSITION OF SECRETIONS

Anal sac secretions of the Mustela species analyzed with TLC showed great similarities, whereas Meles meles, Lutra lutra and Martes martes showed a quite different pattern. No components in the secretions of the latter three species moved when petroleum/ether was used as developer (Fig. 1). GC-analyses (Fig. 2A-C) did not show any of the low molecular weight sulphur compounds typical for Mustela species. This was confirmed by MS-analyses (SIM-technique) (Table 2).

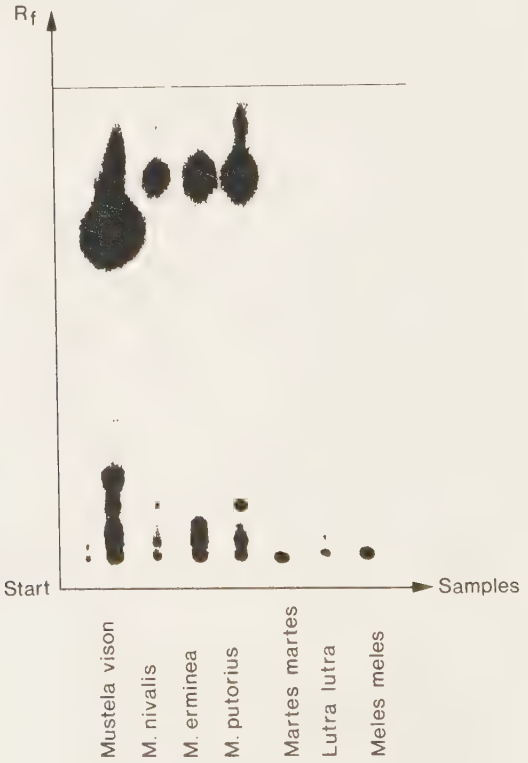

Fig. 1. TLC showing anal sac secretion of Mustela vison, M.nivalis, M.erminea, M.putorius, Martes martes, Lutra lutra and Meles meles with petroleum/ether 90:10 (v/v) as developer.

TABLE 2

Distribution of compounds between investigated species of Mustelidae. Predominant compounds are encircled. A compound not present in amounts over 10 ng/animal is denoted (-). MW = molecular weight.

Peak number	Corresponding compound	MW	Mustela erminea	Mustela nivalis	Mustela putorius	Mustela vison	*Mustela putorius furo	Martes martes	Lutra lutra	Meles meles
1	2,2-dimethylthietane	102	×	-	-	⊗	×	-	-	-
2	2,4-dimethylthietane (cis- or trans)	102	-	×	-	-	-	-	-	-
3	2,3-dimethylthietane (trans-)	102	×	×	×	-	×	-	-	-
4	2,3-dimethylthietane (cis-)	102	-	-	-	-	×	-	-	-
5	2-ethylthietane	102	×	-	-	×	-	-	-	-
6	2-propylthietane, isomers of	116	×	-	⊗	-	-	-	-	-
7	2-propylthietane, isomers of	116	×	-	-	-	-	-	-	-
8	2-propylthietane	116	⊗	-	-	-	×	-	-	-
9	3,3-dimethyl-1,2-dithiacyclopentane	134	-	⊗	×	×	×	-	-	-
10	3-ethyl-1,2-dithiacyclopentane	134	×	-	-	-	-	-	-	-
11	2-pentylthietane	144	×	-	-	-	×	-	-	-
12	3-propyl-1,2-dithiacyclopentane	148	×	-	-	-	×	-	-	-
13	indole	117	×	×	×	×	×	-	-	-
14	o-amino-acetophenone	135	×	⊖	-	-	-	-	-	-
15	benzaldehyde	106	-	-	-	-	-	⊗	-	-

*Data from Crump (1980b)

MELES MELES L., BADGER

The anal sac secretion, preliminary analyzed by means of TLC and GC (Brundin et al., unpubl.), showed volatile esters, fatty acids, cholesterol and cholesterol esters.

isothermal
A
60
50
40
30
20
10
Start
Minutes
B
isothermal
60
50
40
30
20
10
Start
Minutes
isothermal
C
15
60
50
40
30
20
10
Start
Minutes

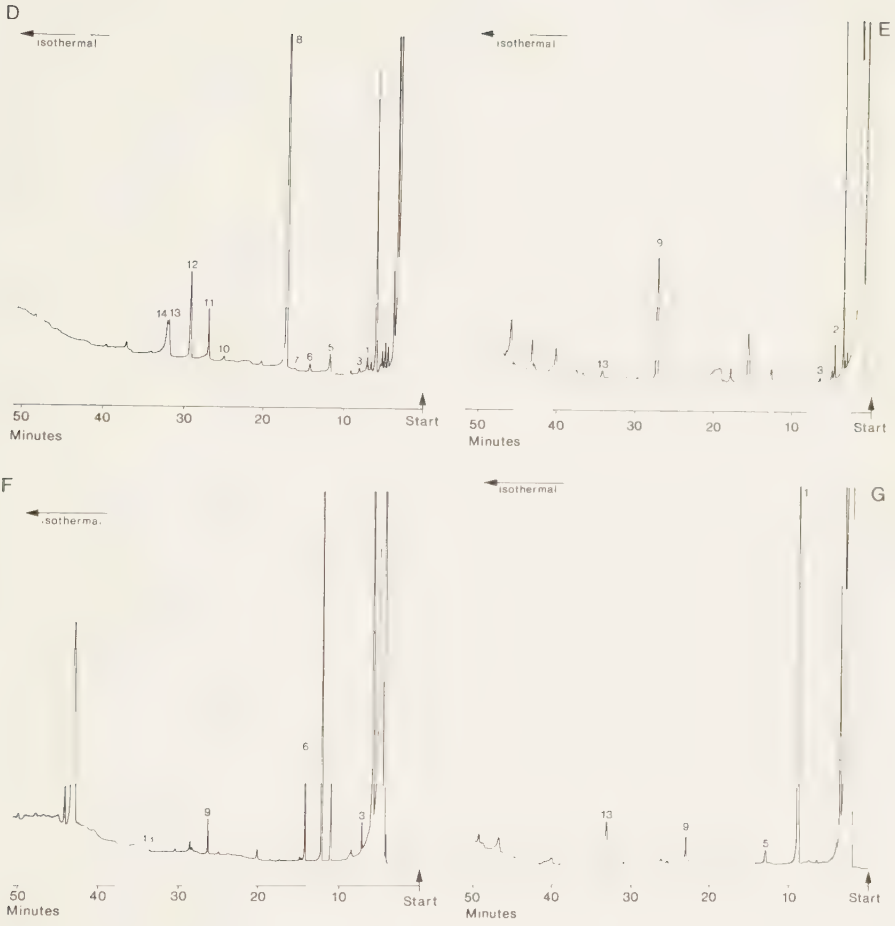

Fig. 2. Gas chromatograms of lower molecular weight components in the anal sac secretion from: A. Meles meles, B. Lutra lutra, C. Martes martes, D. Mustela erminea, E. M.nivalis, F. M.putorius, G. M.vison. Program $4^{\circ}C\ min^{-1}$ ambient - $230^{\circ}C$. Split ratio 1:30. The split was closed 1 min after injection. For A, B and C a 25 m fused silica capillary column (i.d. 0.2 mm) dynamically coated with OV-101 was used. For D, E, F and G a 25 m glass capillary column (i.d. 0.3 mm) with OV-101 as stationary phase was used. Peaks not numbered in C-G are due to artefacts or belong to the solvent.

LUTRA LUTRA L., OTTER

Gorman et al. (1978) analyzed the secretion by means of TLC, using petroleum, diethyl ether and acetic acid (90:10:1 v/v) as developer, and found apocrine gland protein, polymucosaccharide and sebaceous gland lipids.

MARTES MARTES L., PINE MARTEN

The GC showed some low molecular weight compounds and several less volatile components were separated (Fig. 2C). The predominant component, (15), molecular weight 106, had gas chromatographic and mass spectral data identical with those of benzaldehyde. The infrared spectra of the total secretion also indicated the presence of esters by carbonyl absorption at 1740 cm^{-1}.

MUSTELA SPP.

The secretion of the four Mustela species contained lipophilic components, which migrated on the TLC plates (Fig. 1). Gas chromatograms of methylene chloride extracts of these fractions are shown in Fig. 2D-G. Apart from some small individual variations (see Erlinge et al. 1982), the chromatograms of the low molecular weight region were consistent for each species. The chemical composition of the low molecular weight components in the secretions are presented below.

MUSTELA ERMINEA L., STOAT

The secretion of erminea showed the most diverse composition of the examined Mustela species. The GC (Fig. 2D) revealed eleven peaks, some of them previously identified by Crump (1980a), i.e. 2-ethylthietane (5), 2-propylthietane (8) (reported to be the major component), 3-ethyl-1,2-dithiacyclopentane (10), 2-pentylthietane (11), 3-propyl-1,2-dithiacyclopentane (12) and indole (13) (see Table 2).

In addition to these, we detected two isomers, peaks (1) and (3), of the composition $C_5H_{10}S$, (Figs. 3A and C). For peak (1) gas chromatographic and mass spectral data were identical with those of 2,2-dimethylthietane. Peak (3) showed a mass spectrum superimposable with that of authentic trans-2,3-dimethylthietane.

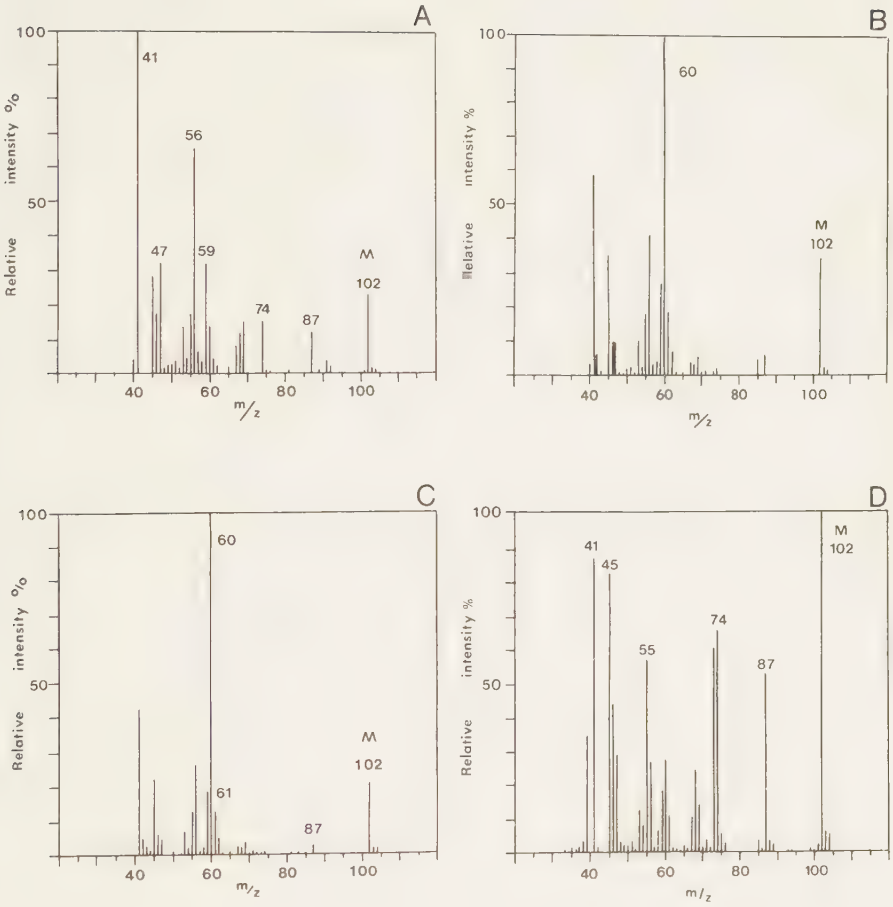

Fig. 1. Mass spectra of the peaks (1), (2), (3) and (5) with the molecular weight 102, illustrated in A, B, C, D, respectively.

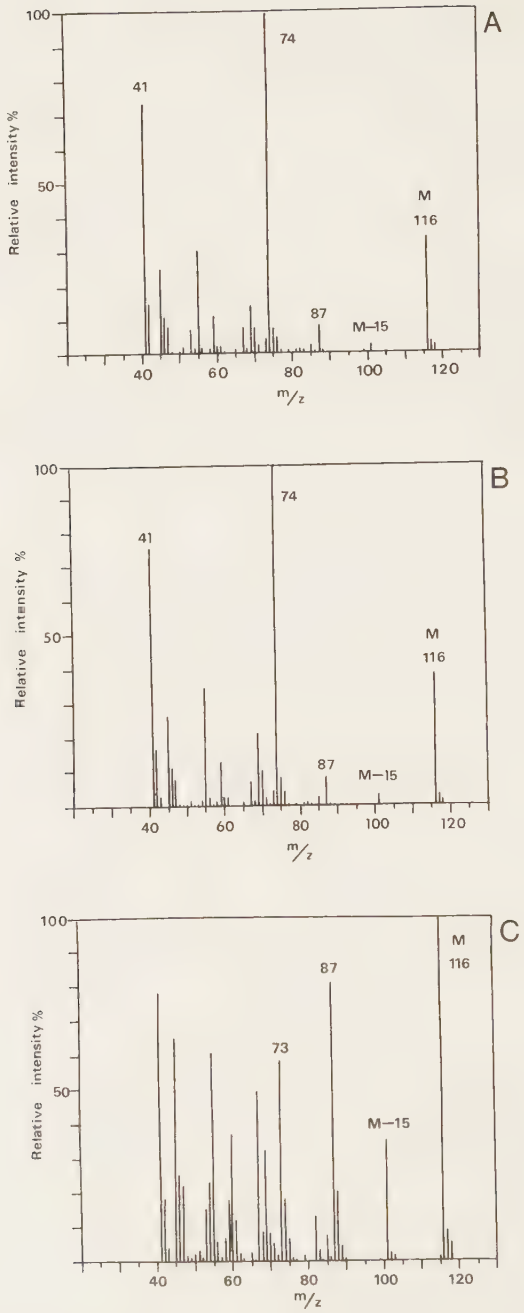

Fig. 4. Mass spectra of the peaks (6), (7) and (8) with the molecular weight 116, illustrated in A, B, C, respectively.

Three components, peaks (6), (7) and (8) (Fig. 4), had molecular weight 116, and were formally isomeric propylthietanes. The mass spectral data of peak (8) (m/z 73) corresponded to a 2-propylthietane (as shown by Crump 1980b), while peaks (6) and (7) (m/z 74) represented thietanes with disubstitution.

Peak (14) (Fig. 5), barely separable from indole on the column used, had a molecular weight of 135. The compound proved to be identical with o-aminoacetophenone as indicated by GC and MS data of an authentic sample (see also Crump 1980a).

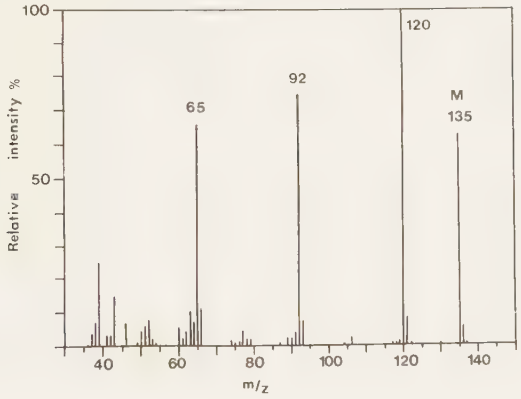

Fig. 5. Mass spectrum of the peak (14) with the molecular weight 135.

MUSTELA NIVALIS L., WEASEL

The volatile portion of the anal sac secretion of nivalis (Fig. 2E) contained fewer low molecular weight compounds than were found for erminea. Peak (2) corresponded to a compound of molecular weight 102 (Fig. 3B), and showed the same GC and MS data as authentic cis- or trans-2,4-dimethylthietane. The MS and GC data corresponding to peak (3) were identical with those of trans-2,3-dimethylthietane from M.erminea (Fig. 3C). Peak (9) (Fig. 6) corresponding to a compound of molecular weight 134, which contained two sulphur atoms as judged from the (M+2) peak. However, the ab-

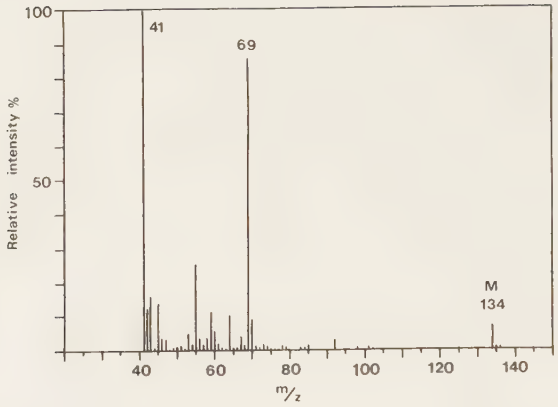

Fig. 6. Mass spectrum of the peak (9) with the molecular weight 134.

sence of a significant peak at m/z M-29 indicated that it could not be 3-ethyl-1,2-dithiapentane. The most likely structure was 3,3-dimethyl-1,2-dithiacyclopentane (Sokolov et al. 1980). Peak (13) corresponded to indole.

MUSTELA PUTORIUS L., POLECAT

Four peaks appeared in the gas chromatogram (Fig. 2F). The mass spectrum of the compound corresponding to peak (3) (Fig. 3C) and comparison of its gas chromatographic data with synthetic compounds revealed it to be trans-2,3-dimethylthietane. Peak (6) proved to be an isomer identical with 2-propylthietane (Fig. 4). The compound corresponding to peak (9) had the same MS data as the dithiacyclopentane of M.nivalis (Fig. 6). Peak (13) was due to indole.

MUSTELA PUTORIUS FURO, FERRET

In Mustela putorius furo, ferret, Crump (1980b) reported 2,2-dimethylthietane (1), 2,3-dimethylthietane (cis- and trans-) (4) and (3), 2-propylthietane (8), 3,3-dimethyl-1,2-dithiacyclopentane (9), 2,3-dimethyl-1,2-dithiacyclopentane (cis- and trans-), 2-pentylthietane (11), 3-propyl-1,2-dithiacyclopentane (12), quinoline and indole (13) (see Table 2).

MUSTELA VISON SCHREBER, AMERICAN MINK

The gas chromatogram for *vison* also showed four prominent peaks (Fig. 2G) corresponding to 2,2-dimethylthietane, peak (1), reported to be the main component in the secretion (Schildknecht et al. 1976, Brinck et al. 1978), 2-ethylthietane, peak (5), and a cyclic disulphide, peak (9), suggested to have the structure 3,3-dimethyl-1,2-dithiacyclopentane (Schildknecht et al. 1976, Sokolov et al. 1980). Peak (13) corresponded to indole.

COMPARISON OF SECRETIONS IN EXAMINED MUSTELIDS

Anal sac secretions of *Meles meles*, *Lutra lutra* and *Martes martes*, each had a characteristic composition of low molecular weight components, clearly distinguished from each other and the *Mustela* species. Several sulphur-containing compounds were found in the *Mustela* species (Table 2). Five different isomeric thietanes with the formula $C_5H_{10}S$ were found, and they were distributed between the species in various combinations and in different quantities. *trans*-2,3-Dimethylthietane (3) was prominent in the secretion of *putorius*, but occurred only to a minor extent in *erminea* and *nivalis*. 2,2-Dimethylthietane (1) and 2-ethylthietane (5) both occurred in *erminea* and *vison*, but (1) was the main component in *vison* (50 % of the volatile fraction), whereas (5) was more pronounced in *erminea*. *trans*-2,4-Dimethylthietane (*cis*- or *trans*-) (2) occurred only in *nivalis*, where it was prominent. Three isomers of 2-propylthietane (6, 7 and 8) appeared in *erminea*, and one of them (8) was the predominant component. The isomer (6) was also found in *putorius*, where it was predominant. 3-Ethyl-1,2-dithiacyclopentane (1) was only found in *erminea*, whereas 3,3-dimethyl-1,2-dithiacyclopentane (9) appeared in the other three species. In addition, two more unidentified isomers of 3,3-dimethyl-1,2-dithiacyclopentane were found in all four species.

Crump (1980b) reported several thietanes and dithiacyclopentanes in secretion of *putorius furo*. Of these only two were found in putorius examined by us.

Two nitrogen containing compounds were found. Indole appeared in all species but o-aminoacetophenone only in *erminea*.

PHYLOGENY OF THE MUSTELIDS

The early evolutionary history of the mustelids is poorly known, but the recent subfamilies Mustelinae (weasels, polecats, martens and others) and Lutrinae (otters) can be traced from the Oligocene, and the subfamily Melinae (badgers) from the Miocene (Romer 1966). During the late Miocene and Pliocene the modern general within the subfamily Mustelinae a.o. Mustela and Martes appeared (Kurtén 1968). Some of the Pliocene forms were intermediate ones between Martes and Mustela (Andersson 1970). Modern species within the genus Mustela are found from the middle Pleiocene (Hall 1951). Phylogenetic relations within the genus Mustela have been discussed from genetic data (Graphodatsky et al. 1976, 1977, Mandahl and Fredga 1980). However, the pattern of karyotype evolution is still unclear (Fredga 1977, Imai and Crozier 1980).

Based on available information on genetic, morphological, behavioural, and ecological characteristics we have constructed a phylogenetic scheme (Fig. 7). The subfamilies represent three different adaptive trends (Sokolov 1968). Pocock (1921) considered the Martes group as a separate subfamily (Martinae), but today Martes is considered as a genus within the subfamily Mustelinae (Ewer 1973). The close relatedness between Mustela erminea and M.nivalis was questioned by Pohl (1910), but recent work by Mandahl and Fredga (1980) and also ecological and behavioural characteristics corroborate the close relationship between the two species. Mustela putorius and M.vison are both distinctly separated from M.erminea and M.nivalis, but M.putorius has more characteristics in common with them.

CONCLUSION

At the generic level the chemical results are in agreement with systematics. All genera have a unique composition of the anal sac secretion, with few, if any, components in common. Species within the genus Mustela on the other hand, showed a very concordant composition of the secretions, although each species had a distinct blend. The secretions contain a very complex mixture of volatile components and with present knowledge it is not possible to determine the degree of relatedness between the species, e.g. erminea and nivalis are considered to be closely related, but their secretions in the low molecular region have less compounds

Fig. 7. Suggested phylogenetic relations of examined mustelids. Chromosome numbers are from Fredga (1967) and Fredga (pers.comm. as concerns Lutra lutra).

in common than have erminea and vison (Table 1). The systematic position of putorius furo in relation to putorius and eversmanni has been much discussed. Cranially putorius furo more closely resembles eversmanni but the karyotype is identical to that of putorius and differs from that of eversmanni (Volobuev et al. 1974, cited in Corbet 1978). Of the examined low molecular weight compounds in putorius furo and putorius secretions only a few were found in common (Table 1).

Intraspecific scent communication apparently is the main function of the anal sac secretion in mustelids (Goethe 1964, Gorman 1980, Erlinge et al. 1982).

Qualitative and quantitative differences in chemical composition between species provide a possibility for interspecific communication. Mustelids do react to scents from congeneric species, as was revealed in behavioural tests with Mustela erminea and M.nivalis (Sandell and Erlinge, unpubl.). The anal sac secretions in small mustelids are also used for defence against predators. Frightened Mustela vison, M.putorius, M.erminea, and M.nivalis were observed to empty their anal sacs (our observations). This function may account for the presence of sulphur--containing compounds.

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Anal pouch secretion in mink Mustela vison

Chemical communication in Mustelidae

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Brinck, C., Gerell, R. and Odham, G. 1978. Anal pouch secretion in mink *Mustela vison*. Chemical communication in Mustelidae. – Oikos 30: 68–75.

The anal pouch secretion from farm mink as well as free living mink was investigated on individual level. A sampling technique using polycthylen catheter allowing collection of secretion from living animals has been worked out. The chemical nature and mass spectra of the four main constituents are described. Two components have been identified as indole and 2,2-dimethylthiacyclobutane. An isomer of the latter has been identified as well as a cyclic disulphide, containing five carbon atoms. A possible structure is 1,2 dithiacycloheptane.

A large proportion of the secretion consists of a large number of chemically very similar compounds of high molecular weight which form a pattern specific for each individual. Comparative studies of the secretions of individuals reveal that this pattern contains individual information. The pattern of the individual appears stable during the whole year in adults, but differs somewhat with juveniles.

No specific sex differences could be found, not even during the reproductive period.

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Секрет анальной железы разводимых и живущих свободно норок исследовался у отдельных индивидов. Были разработаны методы сбора материала с помощью полиэтиленового катетера, дающие возможность собирать секрет от живых животных. Описана химическая природа и масс-спектры 4-х основных компонентов. Два компонента определены как индол и 2,2-диметилтиациклобутан. Был идентифицирован один изомер последнего а также циклодисульфид с 5 атомами углерода. Возможная структура -1,2-дитиациклогептан. Значительная часть секрета состоит из большого числа компонентов, химически очень сходных между собой, с очень высоким молекулярным весом, [illegible] индивидуальные свойства секрета по-вид. стабильны в течение всего года у взрослых животных но у [illegible]ших особей несколько варьируют. Половые различия в секреции не были установлены даже и не в период раз-[illegible].

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1. Introduction

Olfactory communication in mammals has been the subject of several recent reviews (Ewer 1968, Ralls 1971, Eisenberg and Kleiman 1972, Johnson 1973). Most mammals have a highly developed olfactory sense which they employ in social communication, using chemical signals originating in urine, faeces, or cutaneous scent glands. The deposition of the chemical signals on the ground or onto objects in an animal's environment is referred to as scent marking. In the Carnivora anal gland marking is characteristic of viverrids and mustelids. The morphology of the anal glands in mustelids has been described by Schaffer (1940), and Stubbe (1969, 1971).

The anal glands in mustelids comprise two anal pouches, each connected with a complex of cutaneous glands. In some cases there are additional cutaneous glandular areas, viz. in *Meles meles* (L.) a subcaudal pocket and in *Martes* spp. and *Gulo gulo* (L.) glandular patches on the ventral surface of the body. Moreover, in most mustelids there are proctodeal glands which open into the rectum. The glandular structures of the anal region are very complex and several histological types may be distinguished (Schaffer 1940). Ewer (1973) in her classification, however, separates only the almost universal anal sacs proper, the viverrid perineal or perfume glands and the above mentioned circum- or supraanal pouches. Such a simplification may be justified if it is consistent with the function of the gland structures and their exudates. The secretion from the anal sacs is discharged through ducts ending just internal to the anus but separated from the rectum (Fig. 1). At the dilation of the spincter muscle of the rectum the small sac ducts are closed, so that the secretion from the sacs does not come into contact with the faeces. This difference between proctodeal and cutaneous glands is important when discussing the function and the behaviour of scent marking in mustelids.

The chemical composition of the secretion of mammalian scent glands has in no case been fully determined. In mustelids the anal sac secretion of the mink was examined by Schildknecht et al. (1976) and Sokolov et al. (1975). Schildknecht et al. (1976) identified the main component in the secretion of the anal sacs of minks by chemical means and NMR-spectroscopy as 2,2-dimethylthiacyclobutane (mustelan). The structure was confirmed by synthesis. A simple route leading to mustelan was devised by Mayer (1974).

Several kinds of marking can be distinguished on the function of the marks. Marking behaviour is often referred to as "territorial marking" (Heidiger 1949). According to Eisenberg and Kleiman (1972), however, "we must divorce ourselves from considering scent marks as a means of exchanging information, orienting the movements of individuals, and integrating social and reproductive behaviour".

This study is part of a project aiming at analyzing the chemical composition of the secretion from the anal sacs in some mustelid species and experimentally studying the response of the animals to the compounds received. Species of current interest are, beside the mink, polecat *Mustela putorius* L., stoat *M. erminea* L., weasel *M. nivalis* L., badger *Meles meles* L. and pine marten *Martes martes* L. In case of minks, some of Schildknecht's work has been repeated on free flowing secretion from living animals using mass spectrometry in the structure determinations.

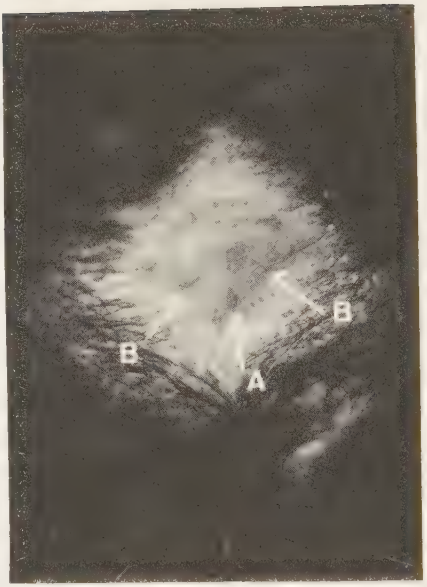


Fig. 1. Anal region in mink *Mustela vison*. A. anus, B. orifices of the ducts discharging the secretion from the anal sacs.

2. Marking behaviour

Territorial behaviour in mink is expressed by the occurrence of monopolized areas, used exclusively by the occupants, and zones of overlapping (Gerell 1970). The balance of the territorial system seems to be maintained by repeated guarding movements covering the whole territory. Marking is performed in at least two ways, a deposition of faeces at discrete loci and gland dragging by rubbing the anal region against objects on the ground. The faeces have a strong odour mainly originating from the exudates of the proctodeal glands. The faeces

are often deposited on prominent terrain features such as rock, stumps, and grassy hillocks (Gerell 1968), probably enhancing the active range of the scent. As the mink territories generally comprise a stretch of a watercourse the zones of contact with conspecifics are very limited in space. The frequency of scats found is higher within these zones compared to the rest of the home range except outside the dens. This was especially observed in subdominant individuals. As intrusions into the territories occurred the scent marks formed by the faeces apparently did not have a deterrent effect. The movements of the intruder, however, were slow and exploratory. Alien scent marks within the territory on the other hand seemed to stimulate the owner of the territory to mark on the point source of the scent. Faeces were often deposited on the top of the traps used in the study.

Active object marking by dragging the anal region against the ground was observed a few times in the field. In captivity minks were often seen marking, especially when introudced into new environments.

The secretion deposited by the anal drag is assumed to come from the anal sacs. The mustelids are able to empty their anal sacs by contracting the muscles around the pouches. This is done during stress situations. When threatened the mink will raise its fur, arch its back, bare its teeth, and move rapidly forth and back. Attacks are accompanied by hissing and highpitched squealing calls. During the back-arch position the tail is uplifted and moved sideways, possibly in order to disperse the strong odour of the anal gland secretion.

3. Material and methods

Keeping of animals
The animals (10 individuals, five of each sex in a known physiological status) were kept and fed in captivity at a mink farm.

Sampling technique
In order to get a pure sample of the secretion from the anal sacs of mink a polyethene catheter, ∅ 1 mm (Portex PP50) was inserted into the duct of the pouch (Fig. 1). The yield of secretion was dependent on the degree of anaesthesia of the animal. If the anaesthesia allowed a muscular activity the secretion flowed from the anal sac via the catheter. The secretion was collected in a small test tube containing a solvent (n-pentane). First a barbiturate drug (Brietal-Sodium) was adminstered intraperitoneally, later to be replaced by ether, which allows a more controlled anaesthesia. The pentane solution was stored at –30°C.

Thin layer chromatography
Analytical TLC was run on commercial silica gel plates (E. Merck) with light petroleum, b.p. 60–85°C-ether 97:3 (v/v) as solvent. Preparative TLC was run on layers with a thickness of 0.5 mm, prepared in the laboratory with Kiesel G (Fluka AG). Solvent as above. The spots were made visible with iodine vapours.

Infrared spectroscopy
The spectra were run on a Pye-Unicam SP 1000 spectrophotometer with $CHCl_3$ as solvent. Path length 0.1 mm.

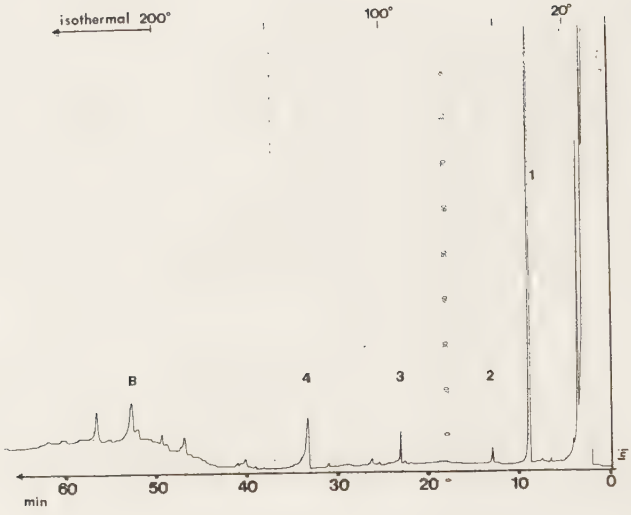
NMR spectra
These were run on a Jeol model JNM FX-60 Fourier Transforming NMR spectrometer operating at 59.8 MHz. About 2000 pulses were collected and $CDCl_3$ was used as solvent. An internal field frequency lock provided by the deuterium resonance present in the solvent was used.

Gas chromatography
Analytical GC was performed on a Perkin-Elmer model 900 gas chromatograph equipped with flame ionization detectors. A 25 m glass capillary column (i.d. 0.3 mm) dynamically coated with OV-101 in the laboratory according to Onuska and Comba (1976) was used. The carrier gas flow was 1.4 ml min^{-1}. Make-up gas corresponding to 25 ml min^{-1} was employed. For the low molecular weight components an injection split giving a ratio of 1:30 was used. The high molecular weight components were studied using the splitter-free intake system described by Ställberg-Stenhagen (1972).

Preparative GC was performed on a Varian model 3700 gas chromatograph equipped with a thermal conductivity detector. The column used had a length of 5 m, i.d. 8 mm, and was packed with 20% BDS on Chromosorb A, 60–80 mesh. The flow of helium carrier gas was approximately 75 ml min^{-1}. The vapours were condensed in solid $CDCl_3$ in narrow U-shaped tubes at liquid nitrogen temperature according to Odham et al. (1970). The yield of recovered material amounted to 70%.

Mass spectrometry
Mass spectra were obtained with a Varian MAT model 112 mass spectrometer at 70 eV and with the ion source operating at 200°C. The spectrometer was connected to a Varian model 1400 gas chromatograph equipped with a 25 ml glass capillary column. The helium carrier gas flow was 2 ml min^{-1} and the splitting ratio at the injection port 1:30.

Hydrogenation experiments
The Wilkinson rhodium catalyst (Osborn et al. 1966) used for the homogenous catalytic hydrogenation of unsaturated compounds containing divalent sulphur by Hörnfeldt et al. (1968) was employed. Six mg of tris (triphenylphosphine)-chlororhodium was dissolved in a degassed mixture consisting of 1.5 ml of benzene and 0.6 ml of hexane. About 0.5 mg of secretion dissolved in 0.5 ml of pentane was added. The solution was vigorously stirred under a continuous stream of hydrogen



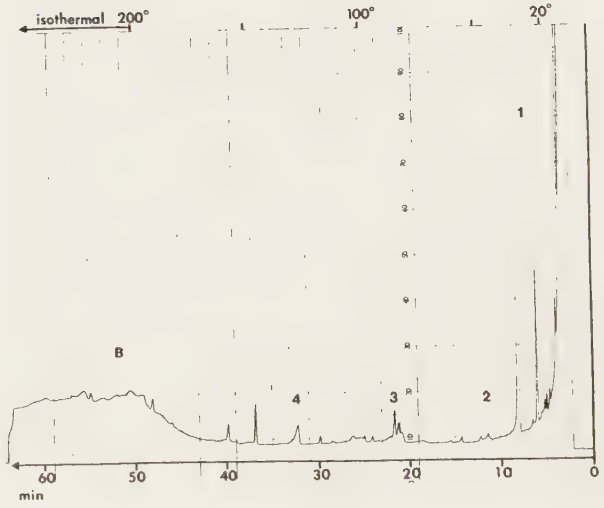


Fig. 2. Gas chromatograms of the lower molecular weight components in the anal pouch from farm mink (above) and free living mink (below). 25 m glass capillary column with OV-101 as stationary phase. Direct injection. Split ratio 1:30. IT 5 min. Program 4°/min ambient – 200°C.

for 3 h at room temperature. The reaction mixture was directly injected into the gas chromatograph.

4. Results

A gas chromatogram of the pentane solution of an anal gland secretion of an animal obtained by chromatography on the capillary columns using direct injection is shown in Fig. 2 (above). In the low molecular weight range four main constituents (1–4) are recognizable. At longer retention times a complex pattern (B) of compounds is seen. The relative proportions of 1–4 and B are estimated to 50, 1, 2, 10 and 37% respectively. Compounds 1–4 will be dealt with in detail below.

The mass spectrum of (1) is reproduced in Fig. 3. It shows a molecular weight (M) of 102. The comparatively large peak at 104 (= M +2) indicates the presence of one atom of sulphur in the molecule. Mass spectra of thiols show ions of considerable abundance at $(M–SH)^+$ and $(M–SH_2)^+$. Such ions are certainly present in the spectrum (*m/e* 68, 69) but only to a limited extent. The formation of abundant fragments at *m/e* 74 involves elimination of ethylene, a process which is known to occur in case of cyclic thioethers (A.P.I. Res. Project 44 catalogue of mass spectral data). Furthermore, such ethers expel SH and SH_2 fragments. The cyclic thioether nature of (1) is also suggested by the very large parent peak. The parent ions readily loose a methyl group giving raise to fragments at *m/e* 87. Subsequent expulsion of ethylene results in ions of *m/e* 59. A large peak of even mass is seen at *m/e* 56. It is most likely formed by elemination of a $CH_2 – S$ molecule after two cleavages α and β to sulphur in the ring system.

A small sample of the pentane solution was shaken with the same volume of an 1% solution of $HgCl_2$ in water and subsequently analysed by GC. Component (1) was unaffected by this treatment. Under identical conditions n-pentanethiol was completely removed by precipitation of the mercuric salt whereas ethyl propyl sulphide remained in solution.

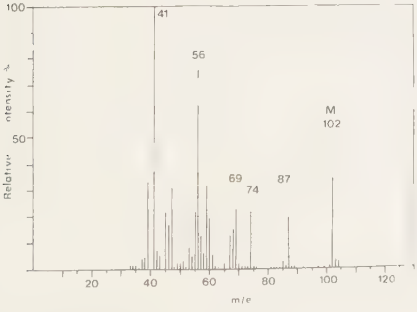


Fig. 3. Mass spectrum of the main component (1), (from the gas chromatogram shown in Fig. 2)

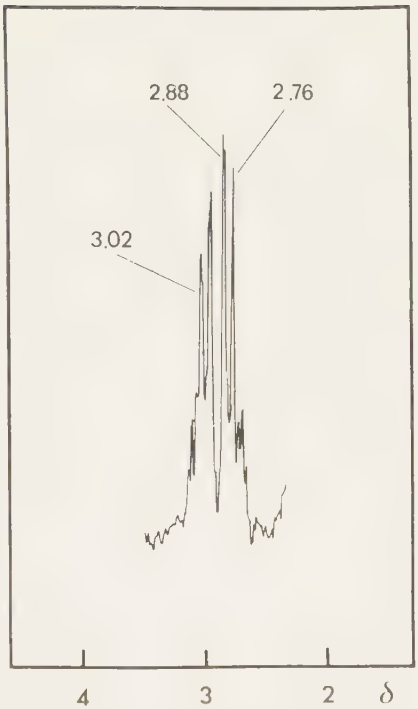
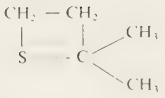


Fig. 4. Part of 60 MHz PMR-spectrum of component (1), (from the gas chromatogram shown in Fig. 2) Jeol Model JNM FX-60 Fourier Transforming. About 2000 pulses were collected. $CDCl_3$ was used as solvent

About 100 µg of gas chromatographically pure (1) needed for NMR-investigations was obtained by preparative GC. The vapours were condensed directly into $CDCl_3$ as described by Odham et al. (1970).

Part of the NMR spectrum is shown in Fig. 4. Two multiplets, corresponding to each 2H are seen at approximately 2.6 and 3.0 ppm. The shifts are consistent with two non-identical methylene units arranged in a ring system. Unfortunately traces of water, present in the solvent obscure the shift region where methyl groups are to be expected.

From the studies by mass- and NMR-spectrometry the most likely structure of (1) is 2,2-dimethylthiacyclobutane.



Fraction (1) is thus identical with mustelan as identified by Schildknecht et al. (1976). The compound was synthesized according to Mayer (1974) and the spectroscopic and other data obtained coincided with those of the natural product.

The mass spectrum of component (2) is shown in Fig. 5. As in case of (1) the molecular weight of the sulphur containing compound is 102 but in many aspects the spectrum is different.

Here the molecular peak constitutes the base peak. Other peaks of large magnitude but negligible in the previous spectrum are observed at *m/e* 45, *m/e* 55 and *m/e* 73. The previously discussed prominent peaks at *m/e* 56, and *m/e* 69 are considerably smaller. A thiacyclobutane structure, isomeric with that of (1) is the obvious choice, particularly when a comparison with spectra of authentic thiacyclopentanes and thiacyclohexane rule out the five- and sixmembered heterocycles. Only comparison with reference material can distinguish between the 2,3-dimethyl- and 2,4-dimethyl-substituted ring structure.

In Fig. 6 the mass spectrum of compound (3) has been reproduced. The molecular peak, at M = 134, is accompanied by abundant fragments at *m/e* 136 (= M+2). The ratio of the (M+2) : M amounts to about 0.09 which implies the presence of two sulphur atoms. The base peak is seen at *m/e* 69 and is most likely formed by loss of HS_2 giving a stable carbonium ion ($C_5H_9^+$). This is known to occure in case of cyclic disulphides (Bowie et al. 1966). As in case of the sulphide (1) the disulphide expells ethylene to give fragments of *m/e* 106. Subsequent loss of HS_2 results in ions of *m/e* 41.

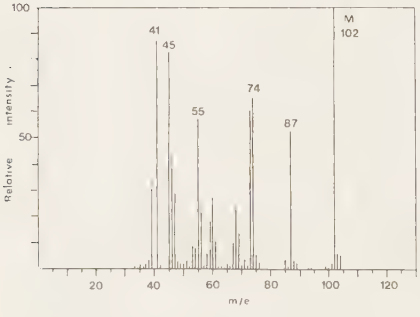


Fig. 5. Mass spectrum of the component (2), (from the gas chromatogram shown in Fig. 2)

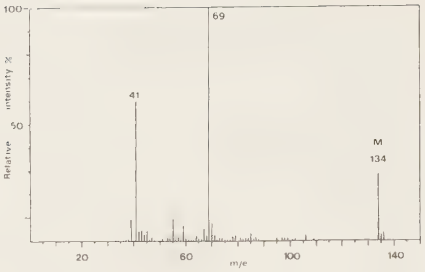


Fig. 6. Mass spectrum of the component (3), (from the gas chromatogram shown in Fig. 2).

Further evidence as to the supposed disulphide nature of the component was obtained from hydrogenation experiments. Hydrogen in the presence of platinum (Adam's catalyst) or rhodium in the form of the Wilkinson catalyst (Hörnfeldt et al. 1968) reduced the component to a more polar compound with considerably longer gas chromatographic retention time.

The absence of a peak at *m/e* 119 (= M–15) in the mass spectrum is noteworthy. A cyclic disulphide containing side chains would be expected to loose a methyl group upon electron impact. A conceivable structure of (3) is therefore 1,2-dithiacycloheptane. Only gas chromatographic and mass spectrometic comparisons with synthetic reference material can prove the structure.

Also Schildknecht et al. (1976) report the presence of a disulphide in the mink anal sac paste. From mass spectrometry and investigations in the UV they conclude that the disulphide is 3,3-dimethyl-1,2-dithiacyclopentane. It is uncertain if this compound is identical with the cyclic disulphide observed in the free flowing secretion of living animals.

Component (4) showed a mass spectrum which indicated a molecular weight of 117. The spectrum was identical with that of authentic indole. Furthermore, when a small sample of the secretion was mixed with indole and subjected to GC-analysis the peak (4) increased. Indole was found also by Schildknecht et al. (1976).

The pentane solution of the secretion was analyzed by TLC. Three main spots could be made visible with iodine vapours. The spots were scraped off and the corresponding material investigated by GC. The fast moving fraction contained the sulphur components previously described and the slow moving proved identical with (4), indole.

The gas chromatogram of the single fraction with an intermediate R_f value indicates a very heterogeneous material consisting of about 10 to 20 compounds. These must accordingly be of a very similar chemical nature. In order to study in more detail the composition and

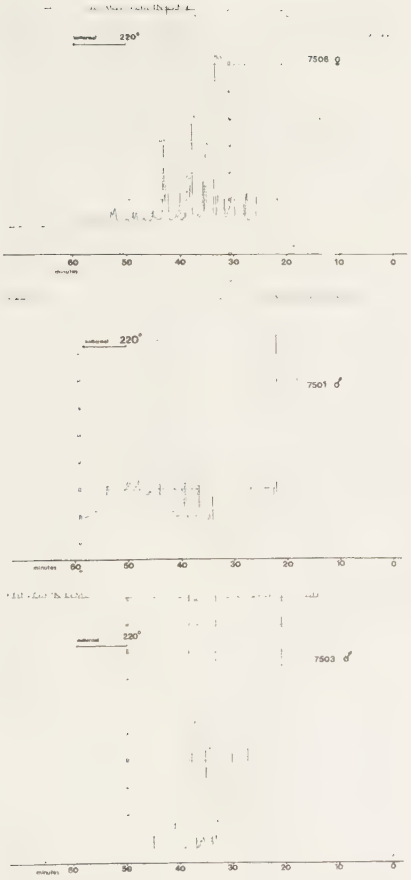


Fig. 7. Gas chromatogram of the higher molecular weight components in the anal pouches from three different minks. 25 m glass capillary column with OV-101 as stationary phase. Splitter free intake system. Program 4/min ambient- 220°C

variation of the fraction by GC (Fig. 7) a splitter free intake system was employed.

The infrared spectrum of the mixture showed strong absorption at 1730 cm^{-1} indicating the presence of carbonyl, e.g. aldehydes and unsaturated esters. Apart from absorption at 1470 cm^{-1} (-CH_2-deformation) a characteristic band is seen at 3010 cm^{-1}, suggesting unsaturation.

5. Discussion

The four main constituents in the low molecular weight range were recorded in the secretion of all specimens studied at all seasons. The four main constituents occur in both free-living and farm minks. So far, we have found more components in the low molecular region in material from the free-living minks (20 specimens) (Fig. 2). The odour of these four compounds is assumed to be indicative of alarm, due to the high volatility of its constituents, its common and permanent presence, and its resemblance with the odour released at fear (as recorded by the human nose). The release of the strong odour of these compounds when the animals are frightened may not only act as a warning signal to conspecifics but also as an antipredator mechanism (Eisenberg and Kleiman 1972).

The complex of compounds (B) in the higher molecular weight range is interesting as far as the patterns are individually different (Fig. 7). The pattern is stable during the whole year in adults but will change somewhat in juveniles. An individual pattern of compounds probably makes it possible for the mink to discriminate between individuals of their own species. Consequently, when a territorial system has been established so that the social status of the individuals involved is fixed and the border of the territories are delimited, each member of the system may be able to record any change in the territorial balance by means of olfactory signals. The recognition of the neighbours without confrontation will stabilize the system and reduce stress, pugnacity, and nonadaptive energy expenditure.

Many authors have stressed the importance of an animal's own scent marks. According to Ewer (1968) it might act to increase its confidence. The motivation of scent marking has also been proposed to depend on the odour field of each individual (Eisenberg and Kleiman 1972). If a disturbance in the odour field occurs, the individual will attempt to restore the previous balance by the release and deposition of its own scent.

No specific sex differences could be seen from the analyses of the anal sac secretion, not even during the reproductive period. In mink, however, defecation and (or) urination often accompany the anal scent marking (MacLennan and Bailey 1969), which gives several possibilities of identifying sex and reproductive state by means of olfactory signals.

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CLASSIFICATION OF INDIVIDUALS IN A POPULATION OF MUSTELA ERMINEA

BY GC/MS

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ABSTRACT

Quantitative variations of selected substances from anal sac secretions were analyzed in a population of killed stoats. Four components of the volatile region and previously identified were used for a statistical analysis (a principal component analysis). A great individual variation occurred especially in the males and concerning the most volatile component A. There was a significant difference between males and females; larger values were obtained for males. No significant difference was found between juvenile and adult males. In shot animals larger values were obtained and the A component predominated the spectrum much more than in animals killed in traps. The latter animals had probably released scent material prior to death. Part of the observed quantitative variations could be referred to as various steps of the biosynthesis of the compounds.

INTRODUCTION

Scent is an important means of communication in mustelids. The anal sacs are specific glands for scent secretion. The chemical composition of the volatile components of the secretions has been examined in Mustela vison (Brinck et al. 1978), in Lutra lutra (Gorman et al. 1978), in Mustela erminea (Crump 1978, 1980a, Erlinge et al. 1982, Brinck et al. in press). Individual variations have been found, indicating that scent might serve in individual identification. The aim of the present investigation was to examine the scent structure in a stoat population. Four compounds of known structure in the anal sac secretion were selected as parameters. Previous studies have demonstrated that the four components are part of an individual pattern which persists through time (Erlinge et al. 1982).

There are several investigations of gland secretion from single individuals, but so far, no investigation has been carried out on the chemical spectrum of gland compounds of a population of a predatory mammalian species. In this study a cross-section of a stoat population and possible differences between individuals of different sex and age are examined.

MATERIAL AND METHODS

ANIMALS EXAMINED

A population of Mustela erminea L. from southern Sweden was studied. The sample consisted of 31 individuals known to sex and

age, viz. 9 females and 22 males killed or trapped. The males were separated into juveniles (animals killed before sexual maturity in February the year after birth, 11 individuals) and adults (sexually mature, 10 individuals). Of the adult males 8 individuals were sexually active as indicated by enlarged testes and 2 were sexually inactive (killed in November-December). One male had been killed in February when entering the puberty. He was classified as a subadult animal.

SAMPLING TECHNIQUE

The contents of the anal sacs were collected as described by Brinck et al. (in press). The organic material was extracted with 0.5 ml of methylene chloride and stored at -20°C until examination. The samples were concentrated to 100 µl. 3 µl was used for each analyses.

GAS CHROMATOGRAPHY (GC)

Analytical GC was performed on Perkin Elmer Model 900 equipped with a flame ionization detector, FID. A 25 m fused silica column (i.d. 0.2 mm) dynamically coated with OV-101 in the laboratory was used. The carrier gas flow was 1.4 ml min^{-1}. A split ratio of 1:30 was used. The split was closed during 60 s after injection. Program 4°C min^{-1} from ambient to -230°C. For further details see Brinck et al. (in press).

MASS SPECTROMETRY (MS)

The compounds were identified by MS, as described by Brinck et al. (in press).

DATA COLLECTION AND HANDLING

The heights in the gas chromatogram of peaks A, B, C and D, corresponding to the components with the chemical structures given in Table 1, were measured manually. The values corresponding to peaks A, B, C and D were used for statistical analysis, carried out with a principal component analysis made with a SIMCA pattern

TABLE 1

Compounds A, B, C and D in anal sac secretion from male and female stoats.
Statistical test: two-tailed t-test.

Compound		♀ totals			♂ totals			P
		N = 9			N = 22			
		$\bar{X} \pm SE$	σ	σ^2	$\bar{X} \pm SE$	σ	σ^2	(two-tailed)
2-propylthietane	A	37.3±12.5	37.6	1253.8	128.5±26.3	123.7	14596.9	<0.01
3-ethyl-1,2-dithiacyclopentane	B	8.6± 1.4	4.2	16.0	13.3± 2.8	13.3	168.9	>0.1 (NS)
2-pentylthietane	C	4.9± 0.8	2.4	5.2	14.9± 2.9	13.5	174.1	<0.01
3-propyl-1,2-dithiacyclopentane	D	7.8± 2.0	6.0	32.0	29.9± 7.6	35.5	1200.8	<0.05

Compounds		♂ sexually active			♂ juveniles			♂ sexually inactive
		N = 8			N = 11			N = 1
		$\bar{X} \pm SE$	σ	σ^2	$\bar{X} \pm SE$	σ	σ^2	X
2-propylthietane	A	79.4±33.4	93.4	7638.7	111.7±3.0	85.0	6572.0	195
3-ethyl-1,2-dithiacyclopentane	B	8.9± 3.3	9.2	74.1	13.2±5.0	16.6	249.4	16
2-pentylthietane	C	15.0± 5.4	18.4	297.0	8.5±1.6	5.2	24.8	13
3-propyl-1,2-dithiacyclopentane	D	27.4±16.2	45.3	1793.7	21.7±5.5	18.2	301.1	14

recognition program on a microcomputer (ABC 80, Luxor Scandia Metric, Stockholm, Sweden) described by Albano et al. (1980). The program was written by S. Wold, University of Umeå, Sweden.

RESULTS

The heights of the four peaks in the volatile region differed greatly (Fig. 1). There was also a considerable individual variation. This was examined by a principal component analysis of the GC spectra of the volatile material components A, B, C and D. According to the relation between the first and second principal components, there was a broad overlap between males and females, but the female sample gathered in a restricted area of the very broad range of the males (Fig. 2). For the second and third principal components the overlap was complete.

In the first plot, the first principal component contained 33 % of all the variability in the material. When two components were treated, the altogether 60 % of the variability was explained (Fig. 2). Principal component three contributed only 12 % to the variability, which can be regarded as a random distribution. There was a high correlation between the variables A, B, C and D. An increase of A was connected with increased values of the other three components.

Two specimens were selected for further analyses (Fig. 3), one adult male and one adult female, both shot in April and having their sample values close to the mean ones. For each of the compounds A, B, C and D the value was larger for the male (relative amounts 152, 19, 10 and 19) each three times larger than for the female (52, 6, 5 and 7). This difference between the sexes was general (Table 1). Statistically significant differences were found for components A, C and D and there was a tendency for a larger amount of component B in males (Table 1).

Comparing juvenile and adult males (sexually active) no significant difference was found in the amount of the four components (Table 1).

When screening the killed males for group distribution of the compounds investigated, it was found that the samples could be divided into two groups. Samples for which compound A had a value larger than 100, were placed in group one. In the second group with A less than 100, compound A was still largest but less predominant. B and C made up a larger part of the composition

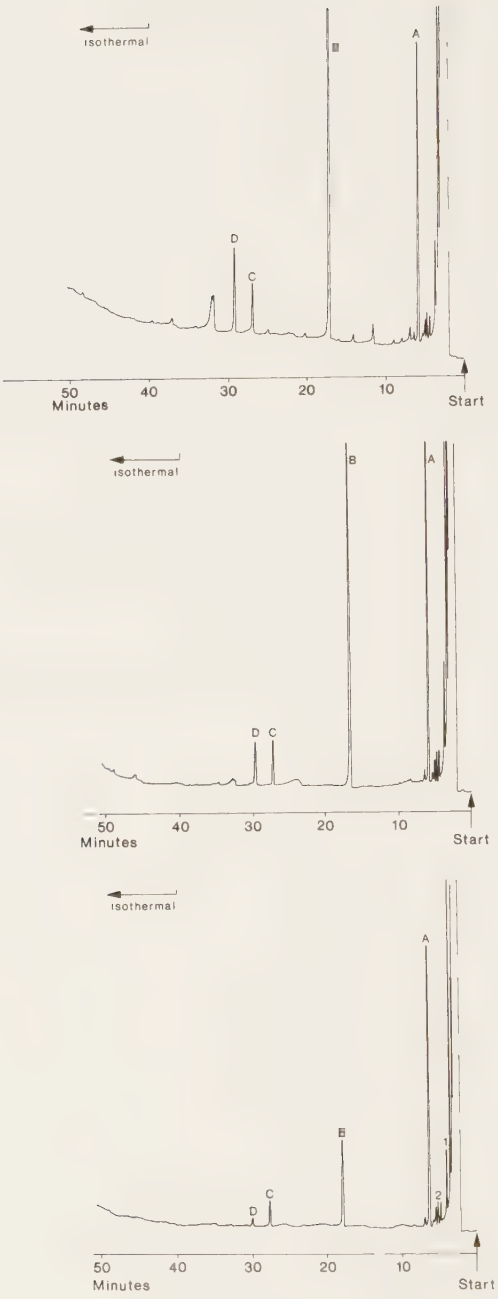

Fig. 1. Gas chromatograms of the lower molecular weight components in the anal sac secretion from three different stoats.

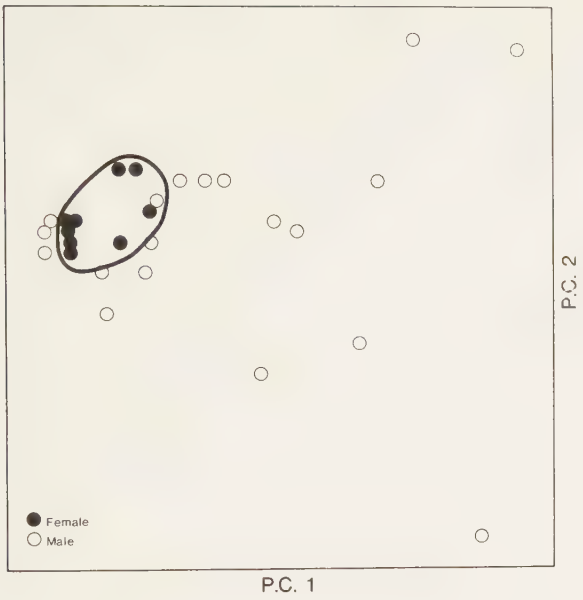

Fig. 2. Plot of the first and second principal components for four selected compounds in the anal sac secretion from male and female stoats.

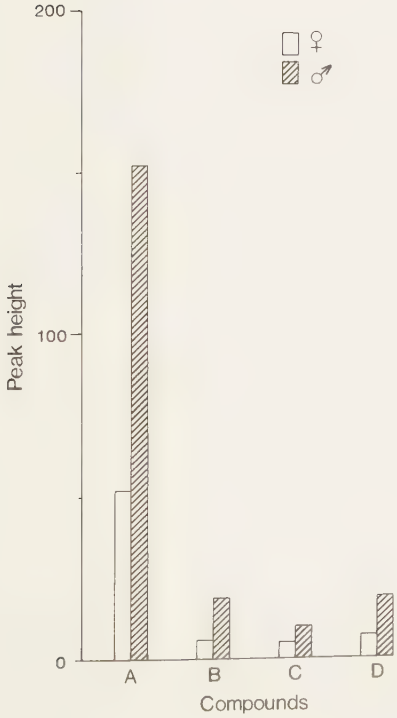

Fig. 3. Histogram showing the relative amounts of the compounds A, B, C and D for one female and one male stoat.

than in group one, while D hardly changed (Fig. 4). The first group consisted of 13 males (7 juveniles and 6 adults) and one female. These animals had been shot. The second group was made up by 10 males (6 juveniles, 1 subadult and 3 adults) and 6 females. They had died in traps and so probably had been stressed before death.

Fig. 4. Histogram showing the relative amount of the compounds A, B, C and D; a) for group 1 with compound A more than 100 and b) for group 2 with compound A less than 100 units.

DISCUSSION

The purpose of the volatile substances of the anal sac secretion probably is manyfold. The main function might be in intraspecific communication, e.g. for individual recognition. Another application could be for defence against predators (Goethe 1964, Gorman 1980, Erlinge et al. 1982). Although no qualitative differences among the volatile substances were found between the sexes, there were clear quantitative differences. As regards the total amount of the compounds, the differences between the sexes may be due to differences in structure/physiology of the glands or the animals' behaviour. There are no consistent differences between males and females as regards frequency of scent-marking, but dominant animals had a higher marking frequency (Erlinge et al. op.cit.).

In the samples, the relative amount of the most volatile component (A) in the secretion varied much more than the amount of the others, and the variation in males was significantly larger than in females. Marking frequency of an individual varies greatly due to a.o. the social environment (Erlinge et al. op. cit.). The variation of the A component in the secretion could be connected with the rate of synthesis of the compounds. This changes the quantitative relations between the components. The biosyntheses of the compounds are not known. Unfortunately, it is not even known if the compounds are racemic or optically active. A change might occur in the mustelid glands and cause part of the variation, related to the emitting of the secretion and the glandular activity. If so, the content of the most volatile components should be high in the glands during storage, while after repeated emptying, the composition of the secretion is different.

A dominant male marks repeatedly, resulting in a high glandular activity, whereas a subordinate animal shows low marking frequency. Thus, the anal sacs of subordinate males may be expected to contain a larger total of the compounds than those of the average male, while a dominant male should usually contain less, due to the high marking frequency. This could explain the high variation of compound A in males. Similarly, the anal sacs of an average juvenile (usually being subordinate) are expected to contain more material than those of an adult male. There was a tendency, although statistically insignificant, for this (Table 1).

in the secretion. A sexually active male marks repeatedly, resulting in a high glandular activity. Thus the glands of the inactive males may be expected to contain a larger total of the compounds than those of the average male, while an active male should usually contain less, due to the high marking frequency. This may explain the high variation of compound A in males. Similarly, the glands of an average juvenile may be expected to contain more material than an active male. There was a tendency, although statistically insignificant, for this (Table 1).

The distribution of the animals / samples into two groups with different compound relations (Fig. 4), supports the hypothesis that the rate of biosynthesis might influence the composition of the secretion. In the samples from group 1 (shot animals), the distribution of the compounds may be more stable, e.g. as a result of storage, while the pattern of the compounds in group 2 (animals killed in traps), seems to represent an unstable phase, e.g. related to the production of the compounds.

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SCENT-MARKING AND ITS TERRITORIAL SIGNIFICANCE IN STOATS, *MUSTELA ERMINEA*

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Abstract. Stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) perform two types of marking behaviour: anal drag and body rubbing. In stoats the two secretions have different chemical composition, and individual differences were found in the anal sac secretion. Dominant male and female stoats marked more frequently than did subordinates. Different messages were probably carried out by the two marks; body rubbing occurred associated with agonistic interactions and seemed to have a threat significance. Anal drag is probably used to impregnate the area with the individual's odour. Scent-marking is considered to be important in allowing assessment of the asymmetry in a conflict, as between a territory owner and an intruder.

Among the solitary carnivores, the commonest form of social organization is one in which a female (or sometimes more than one) occupies a smaller area within a male's larger home range or territory (Ewer 1973). This pattern applies to most of the mustelids (Powell 1979).

Under some conditions, stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) establish a spacing pattern where resident males and females exclude members of their own sex from their ranges (Lockie 1966; Erlinge 1974,1977a; King 1975). In southern Sweden this was so in autumn and winter, especially in linear habitats (hedgerows and ditches). In summer (breeding time) the spacing pattern was less clear and the ranges of males overlapped considerably. Dominance relationships probably developed between males (Erlinge 1977b).

Scent-marking by mammals is thought to be important in the maintenance of territories and in conveying information among individuals in a rank order (Ewer 1973). Marking frequency is correlated with social dominance and the degree of aggressiveness in many mammalian species (Ralls 1971). Field studies on carnivores demonstrate the importance of scent-marking in the maintenance of territories, e.g. in otter, *Lutra lutra* (Erlinge 1968), spotted hyena, *Crocuta crocuta* (Kruuk 1972), wolf, *Canis lupus* (Peters & Mech 1975), European badger, *Meles meles* (Kruuk 1978), and coyote, *Canis latrans* (Barette & Messier 1980; Bowen & McTaggart Cowan 1980).

Marking behaviour involving body rubbing and anal drag have been observed in the weasel (Frank, cited by Goethe 1964), and anal drag has been observed in stoats (Müller 1970). Apart from these notes, no published information is available on scent-marking and its ecological significance in the small mustelids.

This paper describes scent-marking behaviour in stoats and weasels and analyses the chemical composition of the different scent gland secretions in the stoat. It also examines the ecological significance of marking in stoats, especially in the context of territory maintenance, using the perspective of animal conflict theory.

Methods

Observations

Scent-marking behaviour was studied in an enclosure using wild-caught animals. Additional observations were made in the field by snow-tracking and radio-tracking. Animals used in enclosure studies were housed in cages ($1.2 \times 0.5 \times 0.6$ m), usually for less than 3 weeks, and were fed mice. Experiments were performed in two arenas, each 35–40 m^2, containing semi-natural stoat and weasel habitat. The animals were introduced alone or in pairs and marking behaviour was recorded during one or several periods of activity. In paired tests, individuals usually showed either dominant or subordinate behaviour. The following behaviours were used as criteria of dominance: an approach causing submissive vocalization from the animal being approached, lunging and threat vocalization resulting in the other animal fleeing, occupation of a nest, robbery of prey, and chases usually preceded by fights (for details see Erlinge 1977b). Wilcoxon matched-pairs signed-ranks tests were used in the statistical analyses (Siegel 1956), unless otherwise indicated.

Sampling Techniques

Anal gland secretion. Secretions were obtained from 50 stoats both from anaesthetized animals

and from killed and frozen animals. Secretions were collected twice from two individuals. The area around the openings of the anal glands was washed with diethyl ether and the contents of the glands were pressed out on cotton that was previously washed with diethyl ether. The cotton plug was placed in a small test tube and the compounds were dissolved in 0.5 ml of methylene chloride. The extracted samples were frozen until examination.

Skin gland secretion. A 1-cm^2 piece of the fur from five killed stoats was cut from parts of the body used in body rubbing, i.e. belly, cheek, and flanks. The fur was placed in 10-ml ampoule flasks for head-space analyses. The flasks were sealed with rubber stoppers and protected with Teflon sheet and aluminium crimp caps before being heated at 75 C for 15 min.

Gas Chromatography

Analytical gas chromatography with solvent injections was performed using a Perkin Elmer model 900 with FID. We used a 25-m fused silica capillary column (internal diameter 0.19 mm), dynamically coated with OV-101 in the laboratory. The carrier gas flow was 1.4 ml min^{-1}. Make-up gas corresponding to 25 ml min^{-1} was employed. Program: 12 degrees min^{-1} from ambient to 230 C.

For head-space analysis, gas chromatography was performed using a Perkin Elmer model F45 equipped with a unit for automatic head-space analysis and FID detector system. We used a 25-m fused silica capillary column (internal diameter 0.19 mm) with AP 1000 as stationary phase. The carrier gas flow was 1.5 ml min^{-1}. Make-up gas corresponding to 20 ml min^{-1} was used. The injection split ratio was 1:20.

Results

Marking Behaviour

Defaecation and urination. Faeces and urine are probably used in the scent communication of stoats and weasels. Faeces were deposited on particular places, often close to the nest. Some stoats regularly visited defaecation sites when they started and/or finished activity, sniffed the site, and then turned and deposited additional faeces. When moving from the defaecation site, the animal usually performed anal drag. Urination occurred in combination with defaecation.

In addition to defaecation and urination, the scent-marking behaviour of stoats and weasels included anal drag and body rubbing.

Anal drag. When performing anal drag, the pelvis region was pressed against the substrate as the animal moved forwards, or sometimes backwards, with wriggling movements mainly helped by the forelegs. The tail was raised in a curled position. Anal drag was performed on particular places such as stones, tree branches, and bare ground and was often accompanied by urination. The anal glands open just internal to the anus and are separated from the rectum, as in the mink, *Mustela vison* (Brinck et al. 1978). Male and female stoats performed anal drag throughout the year, both inside and outside the breeding season. This was also observed in the field during snow-tracking and radio-tracking. However, in the enclosure studies anal drag marking in male stoats was more frequent in spring, when they were sexually active. Anal drag was performed by sexually immature animals at an age of about two and a half months. At this age they began to move outside the nest (Erlinge, unpublished).

Body rubbing. When performing body rubbing, the belly and the front-lateral parts of the body were rubbed against objects in the environment such as vegetation along runways and on the bare ground. During body rubbing the body was stretched or bent against the object while moving forward. Body rubbing occurred at sites where captured prey were cached, immediately after having deposited the kill. This behaviour was not observed when the cached prey was fetched and brought to the nest for consumption. Body rubbing also occurred when a stoat occupied a nest that had been used previously and impregnated by the scent of another individual, and in close aggressive contacts. It was performed by male and female stoats throughout the year.

Chemical Composition of Gland Secretions in Stoats

The skin gland secretion, which originates from epidermal glands, contains mainly proteinaceous lipophilic compounds of high molecular weight (Quay 1977). These compounds serve as carriers for small molecules with high volatility. The anal gland secretion is more complex and contains predominantly volatile components. Direct gas chromatographic analysis showed more than 200 components with molecular weights less than about 300. The gas chromatographic patterns in the low molecular region differed both quantitatively and qualitatively between individuals and

seemed to be stable in time for each individual (Fig. 1).

The low molecular weight components in the anal sac secretion differed from those found on the fur (Fig. 2). The sulphur compounds characteristic of the anal sac secretion were not found on the fur, although we searched for them using the SIM (single ion monitoring) technique (Odham et al. 1979).

Marking Frequency in Relation to Social Dominance in Stoats

Dominant males and females marked about three times as frequently as did subordinate stoats in paired encounters (Table I; $z = 4.02$, $P < 0.001$). This was consistent in male–male, female–female, and male–female combinations. No consistent difference in marking frequency was found between sexes. The higher marking frequency of dominant animals involved both anal drag and body rubbing. The difference was greater for body rubbing (mean = 2.3 markings/10 min for dominants and 0.5 markings/10 min for subordinates) than in anal drag (means 2.1/10 min and 1.0/10 min respectively). Subordinate individuals had a lower marking frequency when tested in paired encounters with a dominant individual than they did when housed alone (Fig. 3; $z = 3.95$, $P < 0.001$). Dominant individuals increased their marking frequency when in a paired encounter with a subordinate ($z = 2.17$, $P < 0.05$).

Stoats assumed different social status when matched against different individuals (Table II). They always had a higher marking frequency as a dominant than when in a subordinate position (Table II; $z = 2.80$, $P < 0.01$).

Proportion of Body Rubbing to Total Marking in Dominant and Subordinate Stoats

Dominant individuals marked with a higher proportion of body rubbing than did subordinate animals (Fig. 4; $z = 3.01$, $P < 0.01$; Mann–Whitney U-test). The proportion of body rubbing for dominants was greater in paired-encounter tests than when they were alone ($z = 2.01$, $P < 0.05$). Subordinates performed body rubbing at the same low level or slightly but insignificantly less often than when alone ($z = 1.39$, $0.05 < P < 0.10$).

Marking Behaviour Associated with Particular Situations

Body rubbing and anal drag were performed by stoats in different situations (Table III). In paired tests, body rubbing by dominants occurred in association with agonistic contacts with the subordinates. It typically occurred after a dominant had visited the site of an opponent, after fighting and chasing, and after nest occupation. Anal drag was usually performed in combination with defaecation and urination, at places previously marked by anal drag, and at new objects introduced into the enclosure.

Reaction of Subordinate Stoats to Marking Behaviour of Dominants and to Conspecific Scent-marks

In some paired encounters, after an initial period of mutual avoidance, the dominant stoat began intense marking, primarily body rubbing. The subordinate reacted conspicuously, showing signs of fear, giving trill (coo) vocalizations (which serve to mollify the reaction of a conspecific), and attempting to escape from the enclosure. The same occurred when stoats of low social status were introduced alone into a cage previously occupied by a conspecific. They often reacted conspicuously by avoidance or various kinds of fright reactions and signs of uneasiness, when coming in contact with intensely marked places (Table IV). The dominant individuals, on the other hand, did not show any obvious reaction to scent marks apart from marking over previously-marked places.

Discussion

Scent-marking and Social Organization

The stoats showed great individual variation in marking frequency, but no consistent difference appeared between males and females (Table I). The absence of dimorphism in marking is expected in a spacing pattern like that of stoats, i.e. with individual intra-sexual territoriality during most of the year (Erlinge 1977a). Marking dimorphism, on the other hand, is reported in mammals organized in pair or group territories; e.g. in coyotes, a difference was found in marking frequency (Bowen & McTaggart Cowan 1980) and marking has been suggested to have different functions in the two sexes (Wells & Bekoff 1981).

Significance of the Two Marking Behaviours

The secretion found on the fur differed in chemical composition from the anal gland secretion (Fig. 2). Body rubbing and anal drag were used in different situations (Table III). Body rubbing was associated with dominance (Fig. 4): it was typically used in close aggres-

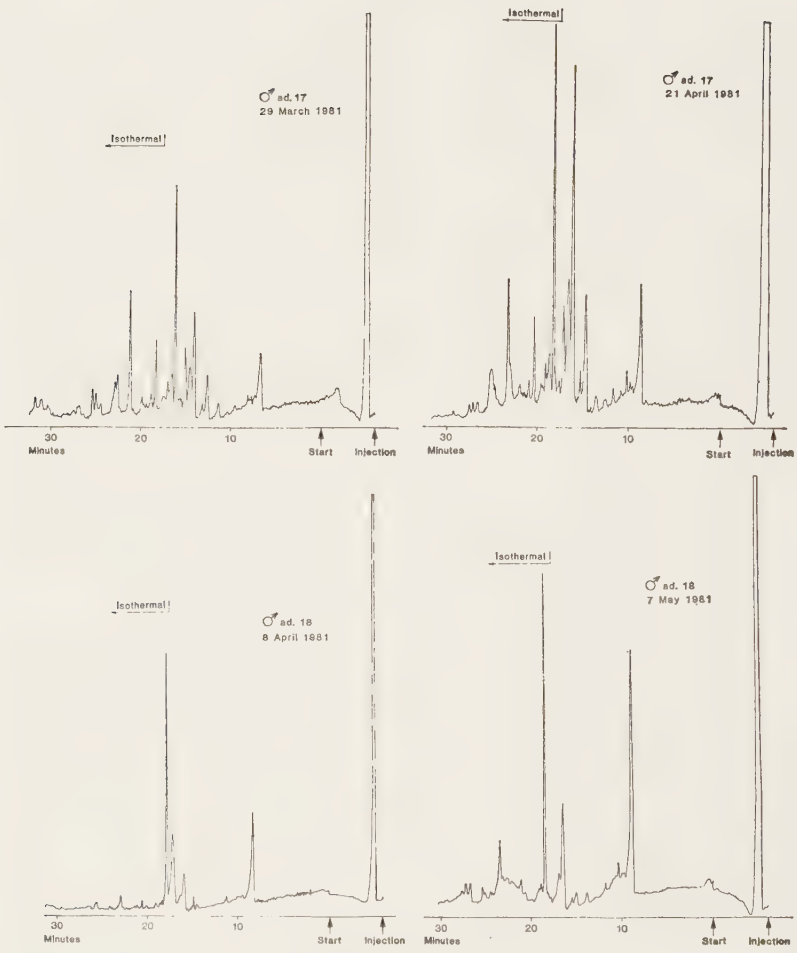

Fig. 1. Gas chromatograms of the lower molecular weight components in the anal secretion from two different male stoats. The secretion was obtained from both animals on two occasions. (25-m fused silica capillary column with OV-101 as stationary phase. Direct injection. Internal time 5 min. Program: 12 degrees/min from ambient to 230 C.)

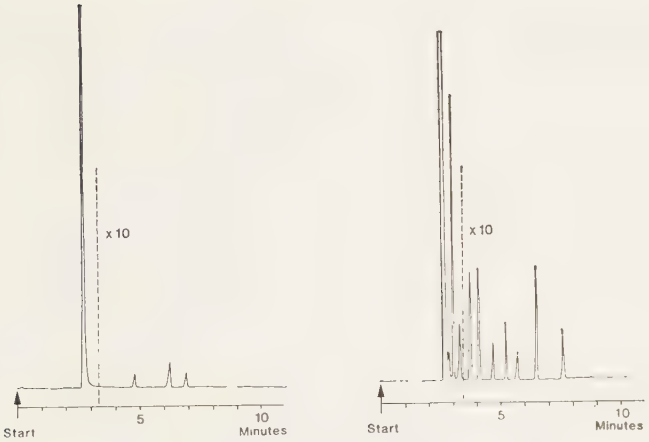

Fig. 2. Head-space gas chromatogram of components from the fur (left) and anal sac secretion (right) from one adult male stoat. The region to the right of the broken line is magnified 10 times. (25-m fused silica capillary column with AP 1000 as stationary phase. Perkin Elmer gas chromatograph equipped with automatic head-space analysis unit. Split ratio 1:20. Isothermal at 117 C.)

Table I. Average Marking Frequency (Number of Markings per 10 min) of Stoats (22 ♂♂ and 12 ♀♀) Relative to their Social Status in Paired Encounters

Tested animals	*N*	Marking frequency (mean ± SE) Dominant	Subordinate	*P*
Female: female	6	5.1 ± 1.08	1.6 ± 0.62	< 0.05 ($T = 0$)
Male: male	16	4.7 ± 0.66	1.4 ± 0.31	< 0.01 ($z = 3.00$)
Male: female*	8	3.3 ± 0.43	1.6 ± 0.90	NS ($T = 7$)
Total	30	4.4 ± 0.44	1.5 ± 0.30	< 0.001 ($z = 4.02$)

*Males are always dominant to females.

sive contacts between conspecifics and seemed to be a threat signal. Anal drag, on the other hand, was used to impregnate the home area, to mark new objects and to mark over the scent marks of conspecifics (Tables III and IV).

The reaction of stoats to scent marks made by conspecifics indicate that individuals may differentiate between their own odour and that of conspecifics (Table IV). The chemical analysis indicated stable individual patterns in the region of highly volatile components of the anal sac secretion (Fig. 1). This may make individual recognition possible.

The different contexts of the two marking behaviours in stoats closely resemble those found in dwarf mongooses (*Helogale undulata*): they marked by cheek-gland rubbing during threat behaviour, whereas the anal gland secretion carried the individual's odour (Rasa 1973).

Scent-marking and Asymmetry Between Individual Stoats

In the stoat, as with mammals in general, conflicts between individuals, e.g. in territory maintenance, are usually settled without fights, but by the avoidance and retreat of one of the participants (Erlinge 1977a). According to conflict theory, non-escalated conflicts occur when there is a large and perceivable asymmetry between the combatants (Parker 1974; Maynard Smith 1974; Maynard Smith & Parker 1976). Scent-marking in stoats is probably an important agent for assessing this asymmetry. Marking frequency was influenced by the animal's

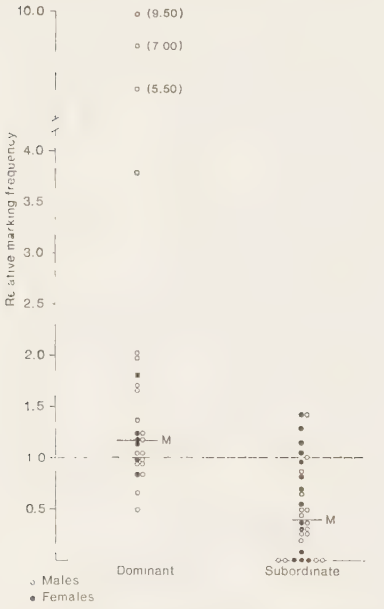

Fig. 3. Change in marking frequency of dominant and subordinate stoats when tested alone and in pairs. The figures show the marking frequencies of paired stoats relative to frequencies when the same individuals were tested alone and given the value 1.0 (broken line). The lines marked 'M' show median values.

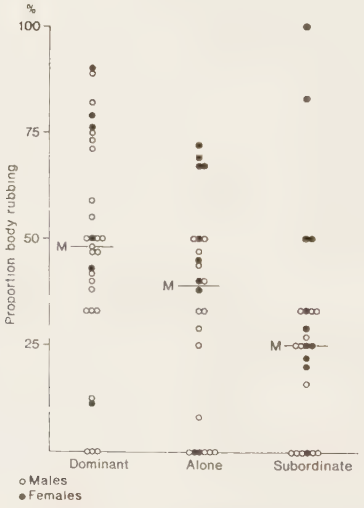

Fig. 4. Proportion of body rubbing (of total markings recorded) in dominant and subordinate stoats tested in pairs and when observed alone. Lines marked 'M' show median values.

Table II. Average Marking Frequency (Number of Markings per 10 min) of Stoats Observed in Both Dominant and Subordinate Situations

		Marking (mean ± SE) frequency when tested animal was:		
Tested animals	*N*	Dominant	Subordinate	*P*
Females	2	5.7 ± 3.30	4.2 ± 2.90	NS
Males	8	3.8 ± 0.77	1.1 ± 0.36	< 0.01 ($T = 0$)
Total	10	4.2 ± 0.82	1.7 ± 0.66	< 0.01 ($z = 2.80$)

self-confidence. Dominant animals had a higher marking frequency (Table I), and they increased their marking rate during encounters compared with when alone, whereas marking in subordinate animals was depressed or inhibited (Fig. 3). There seemed to be a feedback between marking and self-confidence, which further increased the difference in marking rate.

Table III. Situations and Behaviour Associated with Body Rubbing and Anal Drag

Context and behaviour	Observed marking behaviour	
	Body rubbing	Anal drag
Approach by a dominant and marking by this animal immediately after the contact	32	5
Approach followed by attack, fight, and chase. Marking by the dominant followed and sometimes preceded the approach	10	—
Occupation and investigation of nest site previously used by a conspecific	12	—
At sites where captured prey were cached	7	—
At new objects introduced into the enclosure	1	11
Associated with defaecation and/or urination	—	46
At sites previously marked by anal drag	5	49

The figures give the number of tests where the markings were observed. Repeated markings were often recorded during each test.

Table IV. Recorded Reactions to Scent-marks in Tests with Stoats Introduced Alone

Existing circumstances	Reaction	Number of tests
Nest site recently occupied by a conspecific	The stoat avoided the nest site during the exploratory introduction	17
Encountering a nest site (small rodent burrow) previously used by another stoat	The stoat suddenly ran away and took refuge	2
When exploring the enclosure the animal came across a place intensely scented by body rubbing by a conspecific	The stoat gave trill (cooing) or screaming vocalization	5
Encountering a prey (mouse) when exploring the enclosure, which was scent-marked by a conspecific	Abnormal reaction on prey: no catching attempts, fleeing or trill vocalization	5
The enclosure was previously occupied and scent-marked by another stoat	Abnormal behaviour when handling captured prey: the stoat ran for long periods with the prey in its mouth as if to try to escape from the enclosure	6
Encountering scent-marks during the introductory phase	Over-marking scent-marks of conspecific	21

Some observations indicate that scent-marks alone were used to settle conflicts. The stoats in some tests reacted by avoidance, submissive vocalization, and even flight when encountering conspecific scent marks (Table IV).

In the context of territorial maintenance both scent-marking behaviours can be important. By impregnating its home area with anal sac secretion, the territory holder establishes the owner–intruder asymmetry. In interactions with other stoats the territory owner further accentuates the asymmetry using body rubbing.

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MARKING URINE AND PREPUTIAL GLAND SECRETION OF MALE BANK VOLES (CLETHRIONOMYS GLAREOLUS L.); CHEMICAL ANALYSES AND BEHAVIOURAL TESTS

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ABSTRACT

Urine and preputial gland secretion of male bank voles were analyzed by gas chromatography (GC) and mass spectrometry (MS) (selected ion monitoring, SIM). GC/MS analyses showed the presence of n-hexadecyl acetate in preputial gland secretion and in marking urine but not in metabolic urine. Female bank voles responded more strongly to marking urine of males than to metabolic urine and they responded more to preputial gland secretion (pure or added to urine) than to metabolic urine. Dominant males spent more time and marked more frequently as a response to acetate-enriched urine than to controls. The opposite reaction was shown by subordinate males. The results suggest that the acetate functions in bank voles' dominance interactions.

INTRODUCTION

Urine marking behaviour in bank voles and the development of their preputial glands are correlated with sexual maturation (Johnson 1975, Christiansen et al. 1978, Christiansen 1980). Male Clethrionomys voles form stable dominance hierarchies observed both in the field (in Clethrionomys rufocanus, Viitala 1977) and in the laboratory (in Clethrionomys glareolus, Sörensen 1981). In mice (Mus musculus) males demonstrate their dominance through higher urinary marking frequencies (Desjardins et al. 1973) and through qualitative differences in their urine (Jones and Nowell 1974).

In the bank voles, dominant males have larger preputial glands than subordinates (Gustafsson et al. 1980). This suggests a correlation between social dominance and production of preputial gland secretion. Also dominant male bank voles mark significantly more frequently than subordinates when confronted with different types of intruders (Hoffmeyer in prep.). Female bank voles are able to distinguish urine odours of dominant and subordinate males and are more attracted by those of the dominants (Hoffmeyer in press). Christiansen (1976, 1980) suggests that a "sex attractant" of male bank voles is produced by the preputial glands and mixed with the urine as in mice and rats (Bronson 1966, 1976).

The aim of this paper is to relate chemical findings to marking behaviour. The following aspects are studied:

1. The chemical composition of male bank voles' urine and its preputial gland secretion.

2. Reactions of male and female bank voles to urine and preputial secretion of males.

MATERIAL AND METHODS

ANIMALS

The bank voles (Clethrionomys glareolus) were all descendants of a wild stock kept in laboratory (Gustafsson et al. 1980). They were kept in plastic mouse cages (40x20x15 cm) on a photoperiod of 18 hrs light and 6 hrs dark, and at a temperature of 22±5°C. They were sexed at 10 days and weaned at 18-21 days of age, when they were placed in groups of five, males and females in separate cages. All individuals were housed in the same room. This presumably stimulated their sexual maturation. The male urine donors as well as the test animals were selected from all-male groups of 3-5 individuals with stable dominance hierarchies. According to weekly testing they had held the same social status for at least one month prior to the testing. Dominants and subordinates were identified according to the method described by Sörensen (1981).

COLLECTING URINE AND PREPUTIAL GLAND SECRETION

Marking urine was collected on filter paper washed in methylene chloride. Urinations were delineated in UV-light at 254 nm, cut and the paper-pieces were extracted with methylene chloride. The extracts were stored at -20°C.

Metabolic urine was collected in metabolic cages described by Jones et al. (1973), but modified for voles, with separate sleeping, feeding and urination sections. The males were kept individually in the cages overnight. A male donor was used for urine collection at intervals of at least 4 days. The urine was extracted with twice the volume of methylene chloride. The organic fraction was stored at -20°C. Non-treated urine was used either immediately or at the most after 24 hrs at -20°C. Metabolic urine to be compared with marking urine was dropped on filter paper, and then extracted.

Preputial gland secretion from killed specimens was squeezed from the large efferent ducts, and transferred to glass capillaries together with a small amount (40 µl) of methylene chloride.

Collection of markings. The test animal was allowed to mark for 10 min on a glass plate (200x200 mm). The deposited material was extracted with a total amount of 0.5 ml methylene chloride. The solution was concentrated to 10 µl and stored at -20°C until examination.

GAS CHROMATOGRAPHY (GC)

GC was performed on a Perkin Elmer model 900 equipped with a flame ionization detector (FID). A 25 m fused silica capillary column (i.d. 0.20 mm) dynamically coated with OV-101 was used. The injector temperature was 230°C. The split valve was opened 60 s after injection. After another 120 s the column temperature was linearly increased by 4°C min^{-1} from ambient to 220°C.

MASS SPECTROMETRY (MS)

A Ribermag R10-10c quadrupole GC/MS/DA acquisition system equipped with a Carlo Erba Model 4160 GC was used. Separation was performed on a 25 m fused silica column, i.d. 0.2 mm, dynamically coated with SE-54 as stationary phase. One µl samples were injected splitless at an column temperature of 100°C. The split valve was opened 30 s after the injection and the temperature increased linearly by 10° min^{-1} after 2 min to 220°C, where it was held constant. The injector temperature was 230°C. The electron energy used was 25 eV or 70 eV in electron impact ionization (EI) and in chemical ionization (CI). The temperatures in the ion source were 125°C and 100°C, respectively. Ammonia at 1 torr in the ion source served as reactant gas.

RESULTS

CHEMICAL ANALYSES

Gas chromatographic analyses of preputial gland secretions indicated only small amounts of material of high volatility (Fig. 1 a and b). GC retention times of peaks (1) and (2) coincided with those of synthetic n-hexadecyl and n-octadecyl acetate, respectively. Mass spectrometric examination of the volatile material corresponding to peaks (1) and (2) revealed that the fractions contained only minute amounts of acetate. Fig. 2 shows the mass spectrum of a compound isolated from pooled extracts of preputial

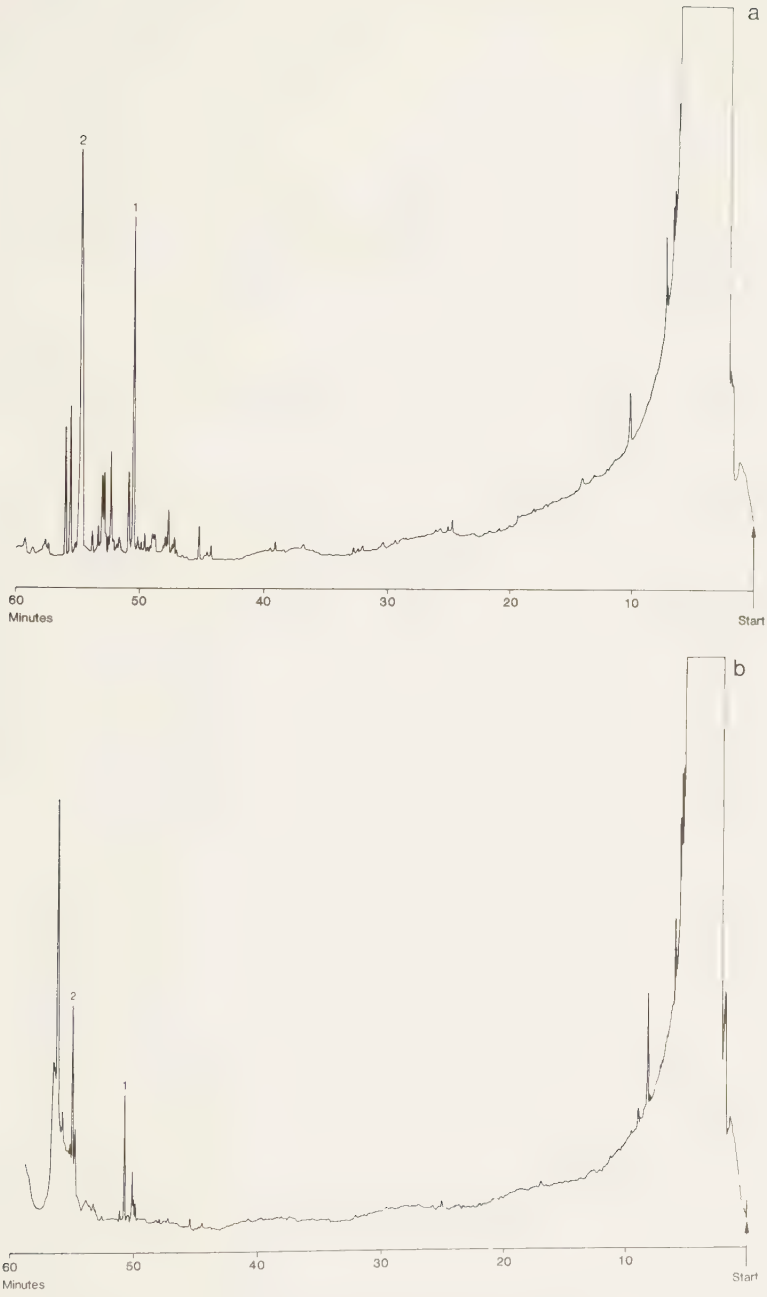

Fig. 1. Gas chromatograms of components in the preputial gland secretion from a) a dominant male bank vole and b) a subordinate male bank vole.

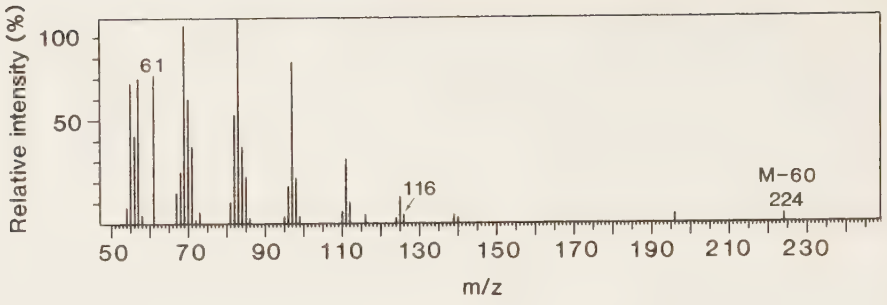

Fig. 2. Mass spectrum of a component isolated from a methylene chloride extract of preputial gland secretion of a dominant male bank vole.

gland secretion of 4 dominant males. The fragmentation pattern was identical with that of authentic n-hexadecyl acetate. The peaks at m/z 61 and m/z 116 are characteristic for saturated acetates (Budzikiewicz et al. 1967). No molecular ions can be seen and the ions of highest mass (at m/z 224) are formed by the loss of acetic acid (M-60) from the molecule. Although we looked intensively for n-octadecyl and n-octadecenyl acetate, we could not find any traces in our samples. Fig. 3 shows a mass fragmentogram of preputial gland secretion from a dominant male, in the region of the GC-retention time for n-hexadecyl acetate, with the mass spectrometer arranged for selected ion monitoring (SIM). Peak (1) represents the integrated signals of ions of m/z 61.0 and peak (2) the signals of ions of m/z 224.2 = M-60, strongly suggesting the presence of n-hexadecyl acetate in the preputial gland secretion. Also, a corresponding fragmentogram of extracts of marking urine indicates the presence of this acetate in the urine (Fig. 4). The same mass fragmentographic analysis was performed also on an extract of metabolic urine from a dominant male. Although a very large volume (8 ml) of urine was extracted, no hexadecyl acetate was detected (Fig. 5 above). Fig. 5 below shows the same extract with 600 pg of authentic acetate added. Interestingly, the fragmentogram (Fig. 5 above) indicated the presence of ions of m/z 224.2 formed by cleavage of a compound with a somewhat shorter GC retention time than that of hexadecyl acetate. Whether these ions reflect the presence of a hexadecyl acetate isomer in the metabolic urine or originate from an entirely different compound is not known.

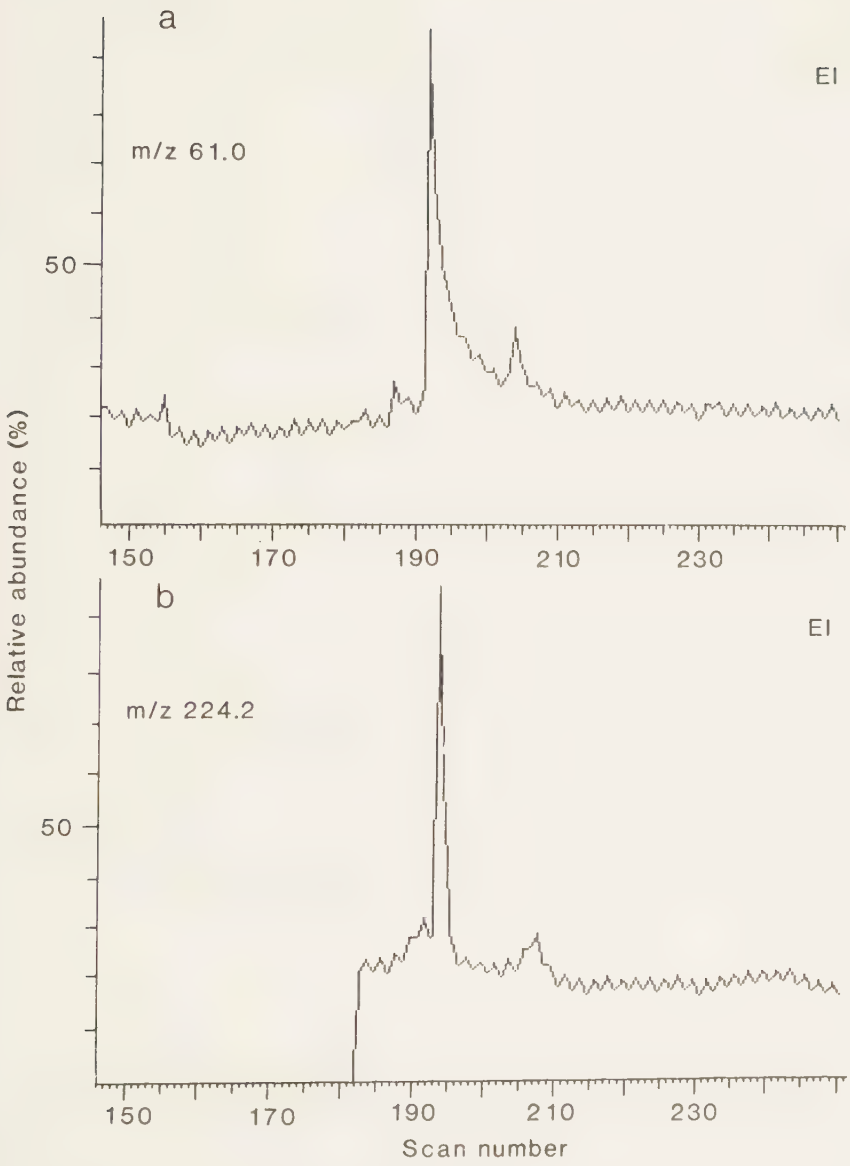

Fig. 3. Mass fragmentograms (SIM) of an extract of preputial gland secret[illegible] of a dominant male bank vole, monitoring of m/z 61.0 and m/[illegible].

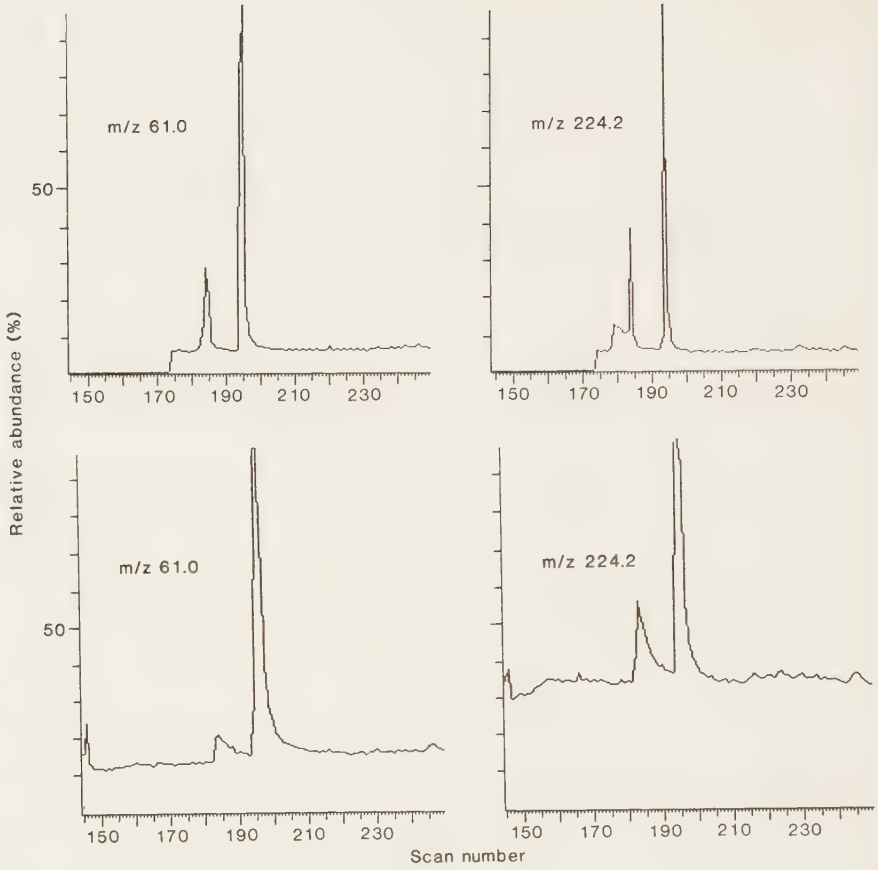

Fig. 4. Mass fragmentograms of a lipid extract of marking urine from one dominant male bank vole (above) and the extract with 600 pg n-hexadecyl acetate added (below). Monitoring as in Fig. 3.

REACTIONS OF BANK VOLES

Female bank voles were more attracted to marking urine than to metabolic urine (Table 1). The females also reacted significantly more frequently to urine supplemented with preputial gland secretion and to pure preputial gland secretion than to metabolic urine. Additional tests were made with dominant males (Table 2). They marked more frequently as a reaction to a mixture of urine and preputial gland secretion than to metabolic urine.

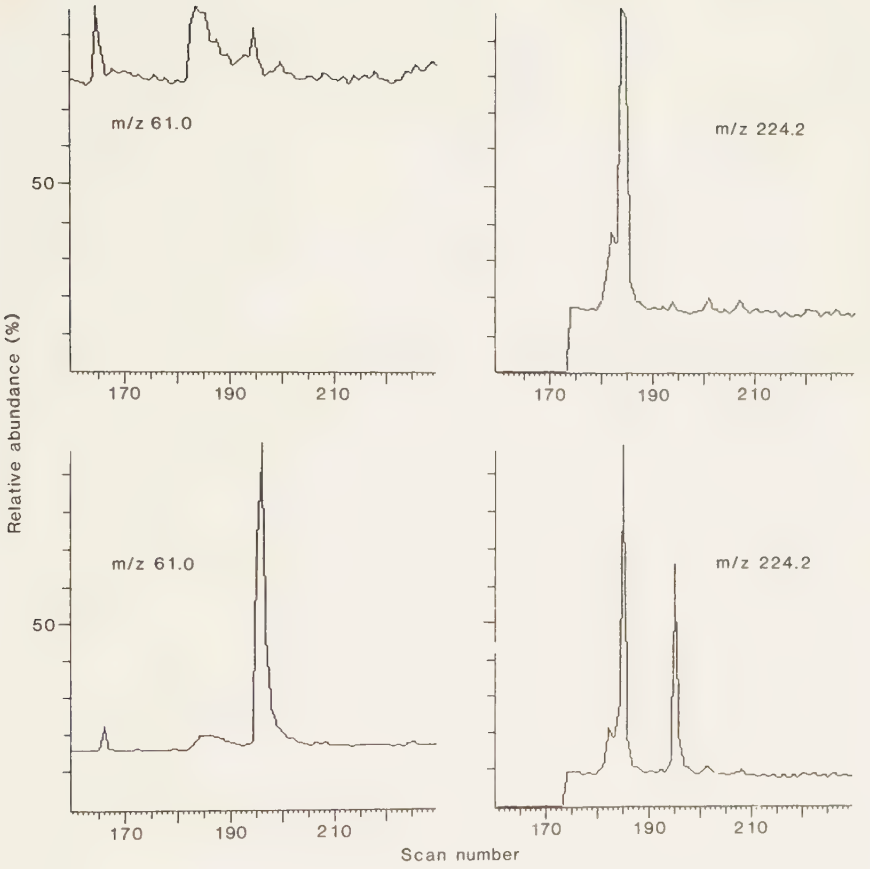

Fig. 5. Mass fragmentograms monitoring as in Fig. 3 of a lipid extract of metabolic urine from one dominant male (above) and same extract with 600 pg n-hexadecyl acetate added (below).

Dominant males in general spent more time with acetate-enriched urine than with controls (Table 3). They also performed urine marking more frequently in areas impregnated with acetate-enriched urine. Conversely the subordinate males spent less time with acetate-enriched urine (Table 3). In females the behaviour was less consistent. The rank of the females was not known and female bank voles generally do not form as distinct dominance hierarchies as males do. The three females reacting positively behaved aggressively within their group, in contrast to the other females.

TABLE 1

Reactions of female bank voles to marking and metabolic urine of conspecific males. The females were tested by a modified olfactometer (Christiansen et al. 1977) in groups of five.

N	Approach Frequency (mean ± SD)		P (one-tailed)
	Marking urine	Metabolic urine	
12	21.3 ± 10.4 (63%)	12.4 ± 8.1 (37%)	< 0.005 (T = 0)
	Urine mixed with Preputial Secretion	Metabolic urine	
12	12.9 ± 6.6 (61%)	8.4 ± 6.3 (39%)	0.005 (T = 3)
	Preputial secretion	Metabolic urine	
12	12.7 ± 8.1 (60%)	8.6 ± 7.8 (40%)	< 0.025 (T = 12.5)

TABLE 2

Urinary marking frequency of dominant male voles exposed to urine mixed with preputial secretion and to metabolic urine of conspecific males.

N	Urinary Marking Frequency (mean ± SD)		P (one-tailed)
	Urine mixed with Preputial Secretion	Metabolic Urine	
12	39.4 ± 20.0 (60%)	26.6 ± 12.9 (40%)	< 0.005 (T = 5)

TABLE 3

Time spent by bank voles in compartment with hexadecyl acetate as compared with controls. The acetate (5 ng) was added to metabolic urine from a dominant stranger.

	N	Animals spending most time with Urine with hexadecyl acetate	Urine
Dominant males	14	13	1
Subordinate males	10	0	10
Females	12	3	9
Total N	36		

DISCUSSION

So far it has not been shown that preputial gland secretion is deposited in connection with urination. If it is so, it is still uncertain whether the secretion is released only in connection with urinary marking. According to Bronson and Caroom (1971), a lipophilic factor, strongly attractive to sexually experienced female mice was found in a preputial gland homogenate and externally voided urine, but not in bladder urine or urine from preputial-ectomized males. Bronson (1979) concluded that preputial secretions probably are mixed with urine during marking. For rats Gawienowski et al. (1976) suggested that a marking pheromone was produced by an "androgen controlled gland" and released in the urine. Similar suggestions were made about the bank vole by Christiansen (1980).

The results of this study show that preputial secretion is connected with urinary marking. First, hexadecyl acetate is present in the preputial gland secretion and is deposited in marking urine, but not in metabolic urine. Second, both males and females react strongly to urine mixed with preputial gland secretion when this mixture is presented as an alternative to metabolic urine.

Only aggressive/dominant individuals reacted positively to the hexadecyl acetate. This suggests that the acetate has a function in dominance interactions. Furthermore, dominant males mark more frequently than subordinates (Hoffmeyer in prep.) and have larger preputial glands (Gustafsson et al. op.cit.).

In male mice testosterone support is necessary for marking behaviour (Bronson 1979). The same appears to be the case for bank voles (Christiansen 1980). Testosterone treatment of subordinate male bank voles caused a change in the histology of the preputial glands and an increase of the amount of hexadecyl acetate. The marking behaviour and social status did not change during the period of testosterone administration (Brinck et al. in prep.). In mice, subordinate individuals remain unchanged also when they bear implants of testosterone (Bronson 1976).

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EFFECT OF TESTOSTERONE ON THE STRUCTURE AND SECRETION OF THE PREPUTIAL GLANDS IN SUBORDINATE MALE BANK VOLES, CLETHRIONOMYS GLAREOLUS

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ABSTRACT

Subordinate male bank voles (Clethrionomys glareolus) were treated with testosterone to determine its effects on their preputial glands. Testosterone caused hypertrophy of the secretory cells and light microscopy revealed a large increase in the amount of granules and lipid droplets within the glands. Gas chromatographic and mass spectrometric assays confirmed these observations; the lipid fractions of secretion from testosterone-treated specimens contained increased amounts of hexadecyl acetate compared to untreated ones. Testosterone treatment and subsequent effects, however, did not alter the social position of the subordinate animals.

INTRODUCTION

Preputial glands are found in many small rodent species (Brown and Williams 1972). They are modified sebaceous glands, on both sides of the penis base in males and on both sides of the pelvic base in females.

In mice and rats the preputial glands play a role in chemical communication (Bronson 1971, Brown and Williams 1972). Chemical analyses have shown that preputial secretion contains an aliphatic acetate, n-hexadecyl acetate, which is deposited by male bank voles during urinary marking ("marking urine") (Brinck and Hoffmeyer unpubl.). The size of the preputial glands varied with the males' social status, and scents originating from the glands appeared to be essential for social communication (Brinck and Hoffmeyer op.cit.).

In the mouse, the preputial glands of males contain relatively higher amounts of aliphatic acetates than do female glands, and the amounts of the acetates can be elevated by testosterone injections (Spener et al. 1969, Sansone-Bazzano et al. 1972). Furthermore, dominant males have larger preputial glands than subordinate males (Bronson and Marsden 1973 regarding mice, Gustafsson et al. 1980 regarding bank voles) and testosterone levels are higher than in subordinates (McKinney and Desjardins 1973).

The aim of the present study is to investigate whether testosterone treatment would influence (i) the histology of the preputial glands and (ii) the amount of hexadecyl acetate in secretions from subordinate male bank voles.

MATERIAL AND METHODS

ANIMALS AND TREATMENT

Adult male Clethrionomys glareolus from a laboratory colony (cf. Gustafsson et al. 1980) were used. The animals were kept in standard plastic mouse cages (40x20x15 cm) with a wire mesh cover at a photoperiod of 18 h light / 6 h dark and a temperature of 22±5°C. Wood shavings were used as bedding. The animals were allowed water and a commercial mouse food ad libitum, supplemented once a week with standard rabbit- and guinea pig--pellets.

After weaning, at 18 days of age, they were placed in single--sex groups of 5, all housed in the same room.

The males selected for treatment were subordinates, from single-sex groups with stable dominance hierarchies (i.e. groups in which a dominant male had been identified, as described by Sörensen (1981) for at least 1 month previous to the start of this experiment).

Five subordinate males, from five different male groups, were used. Four days before starting the experiment each male was placed in a separate cage. (One dominant male served as control.)

PREPUTIAL GLAND-ECTOMY AND PREPARATION OF GLANDULAR EXTRACTS

On the first day of the experiment the animals were unilaterally preputial gland-ectomized (the side of ectomy was randomly chosen). Secretion was squeezed from the large efferent duct and inserted into a glass capillary with 50 µl of methylene chloride. The capillary was fused and stored at -20°C. The remaining part of each (ectomized) gland was prepared for histological studies. Each untreated gland served as a control for the remaining gland.

Before the chemical analyses the extracted glandular tissue was removed from the solution. The individual samples were concentrated until dryness. Each sample was diluted with 10 µl hexane for quantification.

Hormone injections. Daily injections (0.1 ml/20 g b.w.) of testosterone-propionate at 3 mg ml^{-1} in sesame oil were given subcutaneously on the shoulder. The injections started the day after ectomy and continued for 10 days.

HISTOLOGICAL TECHNIQUE

The animals were autopsied the day after the last injection. The secretion of the remaining gland was taken for chemical analyses and treated as described above. The reproductive organs were dissected and weighed fresh. All preputial glands were fixed in Bouin´s fluid, sectioned at 7 µm, and stained with a tetrachrome combination (Borg et al. 1978). The sections were studied anonymously by light microscope.

GAS CHROMATOGRAPHY / MASS SPECTROMETRY (GC/MS)

A Ribermag R10-10c quadrupole GC/MS/DA system equipped with a Carlo Erba Model 4160 GC was used. Separation was performed on a 25 m fused silica column, i.d. 0.2 mm, dynamically coated with SE-54 (Alltech) as stationary phase. One µl samples were injected splitless at an injector temperature of 230°C and a column temperature of 100°C. The split valve was opened 30 s after the injection and the temperature increased linearly by 10°/min after 2 min to 220°C, where it was held constant. The electron energy used in EI was 25 eV or 70 eV and in CI 70 eV. The temperatures in the ion source were 125°C and 110°C. Ammonia at 1 torr in the source served as reactant gas. The synthetic hexadecyl acetate used was from Larodan Lipids, Malmö, Sweden.

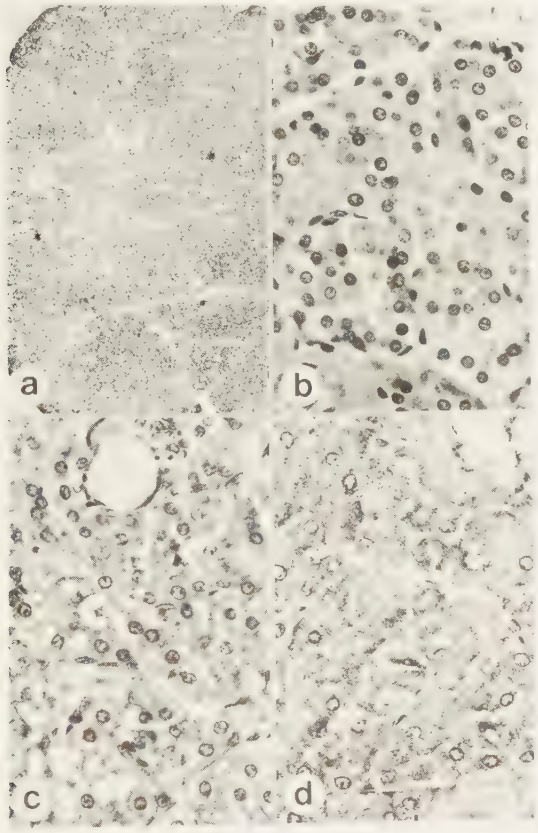
For head space analyses, GC was carried out using a Perkin Elmer Model F45 equipped with a unit for automatic injections and a FID detector system. A 25 m fused silica column (i.d. 0.19 mm) with AT 1000 (Alltech Ass., Deerfield, Ill.) as stationary phase was used. The carrier gas flow was 1.5 ml min^{-1}: Make-up gas corresponding to 20 ml min^{-1} was used. The injection split ratio was 1:20.

RESULTS

HISTOLOGY

The surface was covered with connective tissue from which interstitial tissue passed to the interior to subdivide the gland into lobules (Fig. 1a). Further subdivisions of these lobules by interstitial tissue formed groups of cells of either granule or lipid producing type. The two types were mingled within the gland in untreated specimens and they were hard to distinguish between

Fig. 1a. A preputial gland from an untreated subordinate male bank vole, showing the division into lobules by insterstitial tissue. X38.
b. A magnification of the gland in Fig. 1a, showing the scarce content of granules and lipid droplets. X370.
c. A preputial gland from a subordinate male bank vole, treated with testosterone, showing a large vesicle and a region with lots of lipid droplets. X370.
d. The same gland as in Fig. 1c, showing another region with large amounts of granules.



when there were almost no lipid droplets present and only a few scattered granules. The cytoplasm of the lipid producing cells was, however, darker (Fig. 1b). Large vesicles (average 30 µm) were scattered within the gland (Fig. 1c), the amount differing between individuals. The vesicles were surrounded by a simple layer of flattened cells.

The testosterone treatment changed the histological appearance; the cells in the gland hypertrophied. The amount of granules and lipid vacuoles increased dramatically (Fig. 1d) and were scattered

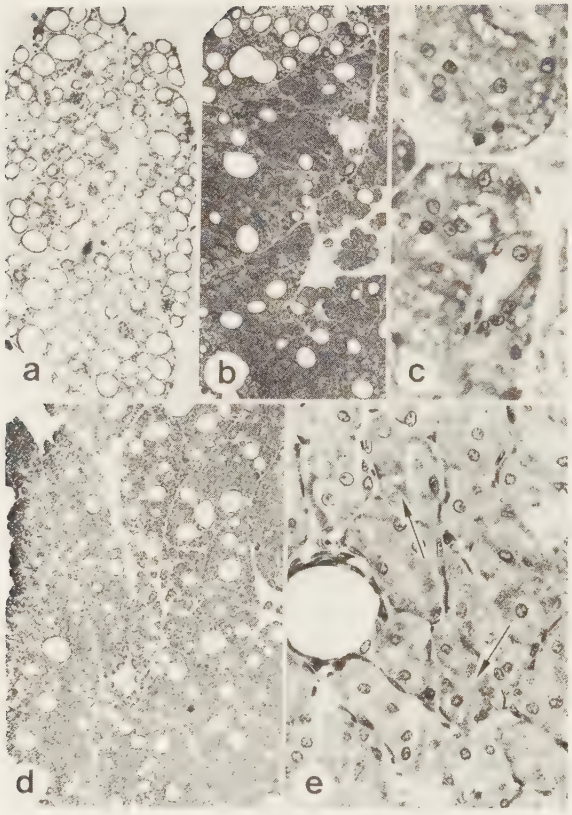


Fig. 2a. A preputial gland from an untreated subordinate male bank vole, with an extremely high amount of large vesicles. Note the empty-looking interspatial tissue. X38.

b. A preputial gland from the same specimen as in Fig. 2a after hormone treatment. The interspatial tissue has developed into a more "normal" appearance. X38.

c. A magnification of the gland in Fig. 2b, showing the large amount of lipid droplets, clearly dominating over the granule cell-type. X370.

d. A preputial gland from a dominant male bank vole. X38.

e. A magnificantion of the gland in Fig. 2d. Between the many large vesicles the tissue consists mostly of groups of granule cells. Apart from the subordinate anaimals, whether hormone treated or not, lipid droplets were found also within the granule cells (arrows). X370.

over the cytoplasm. The number of vesicles did not change after hormone treatment, nor did their content of secretion. One specimen differed from the others both prior to and after treatment. Before treatment its gland was filled with large vesicles (Fig. 2a) and the interspatial tissue was empty of structures. After

treatment the amount of vesicles decreased but still was much greater than in any of the other specimens (Fig. 2b). The tissue between the vesicles had changed to a "normal" appearance with hypertrophied cells of the two types. There were more lipid droplets, however, than granule cells.

The histological picture of a preputial gland taken from a dominant male revealed a high density of large vesicles and the intervening tissue consisted almost entirely of granule cell groups. However, a few lipid droplets could occasionally be seen within the granule cells (Fig. 2c). All the vesicles were filled with secretion.

CHEMICAL ANALYSES

The preputial gland secretion contained mainly lipophilic compounds of medium or high molecular weight. Head-space as well as direct gas chromatographic analyses did not show any material of high volatility (Fig. 3). N-hexadecyl acetate was earlier identified by GC/MS in preputial gland secretion in bank voles (Brinck and Hoffmeyer, unpubl.). Mass fragmentogram (single ion monitoring) of hexane extracts from preputial gland secretions of five individual specimens are shown in Figs 4 and 5. Peak (a) corre-

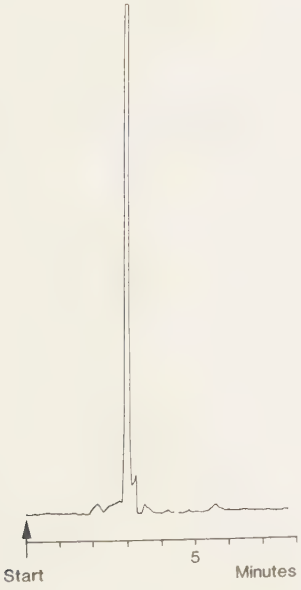


Fig. 3. Head space chromatogram of components from preputial gland secretion from one dominant male bank vole. 25 m fused silica capillary column with AP 1000 as stationary phase. Perkin Elmer gas chromatograph equipped with automatic head space analysis unit. Split ratio 1:20. Isothermal at 117° C.

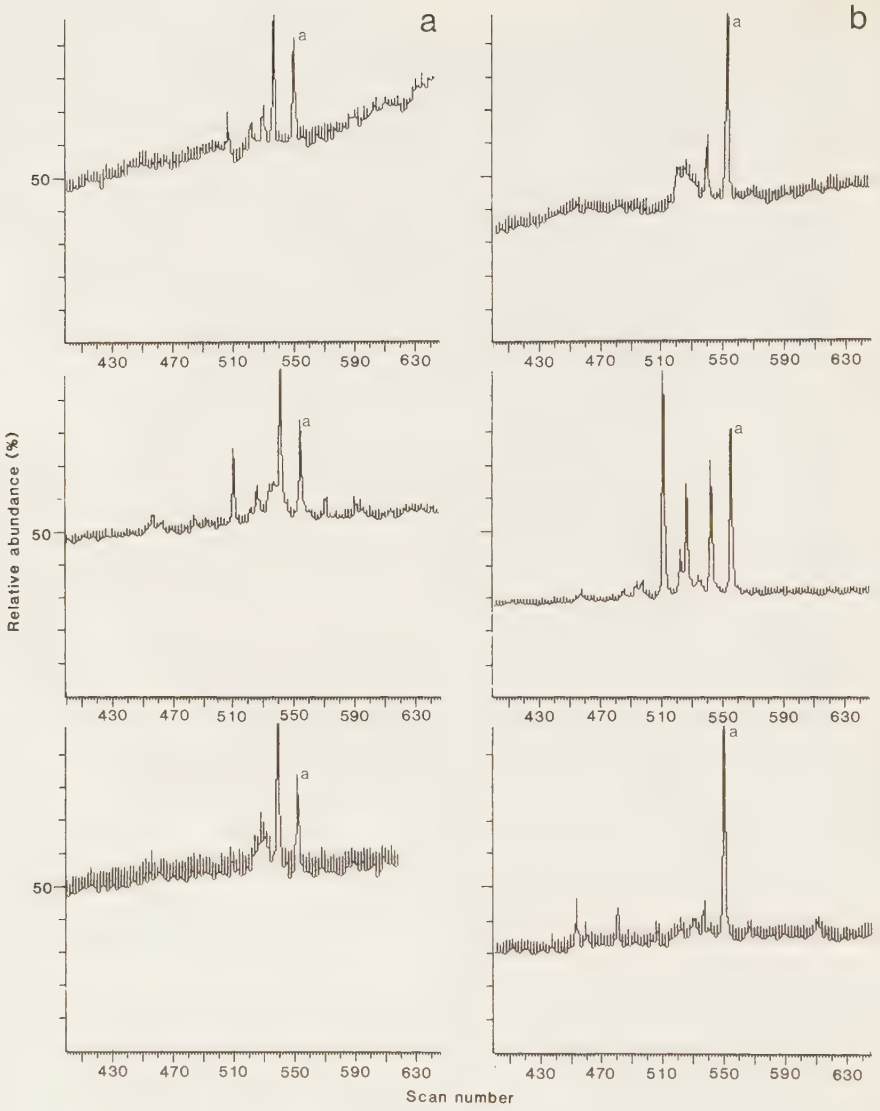


Fig. 4. Mass fragmentograms of lipid extracts from preputial gland secretion from subordinate male bank voles, specimens numbered 1, 2 and 3, a) before testosterone treatment and b) after hormone administration. Peak (a) corresponds to the (M+18) adduct of n-hexadecyl acetate by chemical ionization with NH_3 as reactant gas.

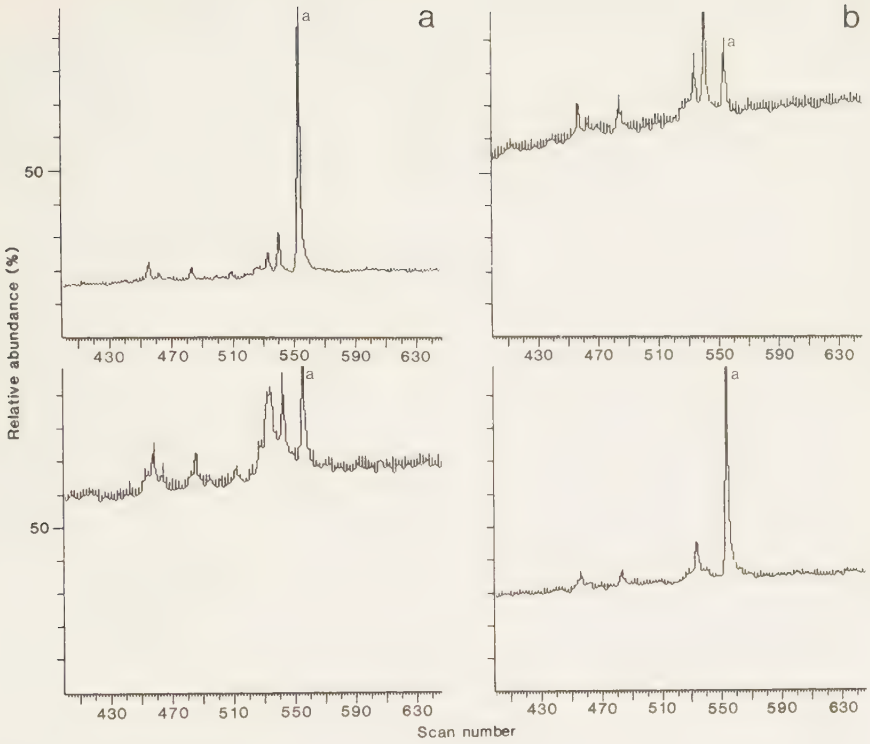


Fig. 5. Mass fragmentogram of lipid extracts from specimens numbered 4 and 5 a) before testosterone treatment and b) after hormone administration. Peak (a) corresponds to the (M+18) adduct of n-hexadecyl acetate by chemical ionization with NH_3 as reactant gas.

sponds to the (M+18) adduct of n-hexadecyl acetate (M=284.5) by chemical ionization with NH_3 as reactant gas. The integrated signals from the (M+18) ions (peak (a)), in the mass fragmentograms from untreated glands, were always lower than the corresponding treated one. All treated individuals exhibited an increased acetate content after testosterone treatment (Table 1).

In a mass fragmentogram (single ion monitoring) (Fig. 6) of components in preputial gland secretion from a dominant male, peaks (1) and (2) corresponded to adducts of hexadecyl acetate fragments of (m/z 61.0) forming the base peak in spectra from saturated acetates and of m/z 224.2 (M-60) resulting from the loss of acetic acid (Beynon et al. 1961). The amount of hexadecyl acetate (530 ng/gland) was estimated by comparison of its ion intensities with those of synthetic hexadecyl acetate.

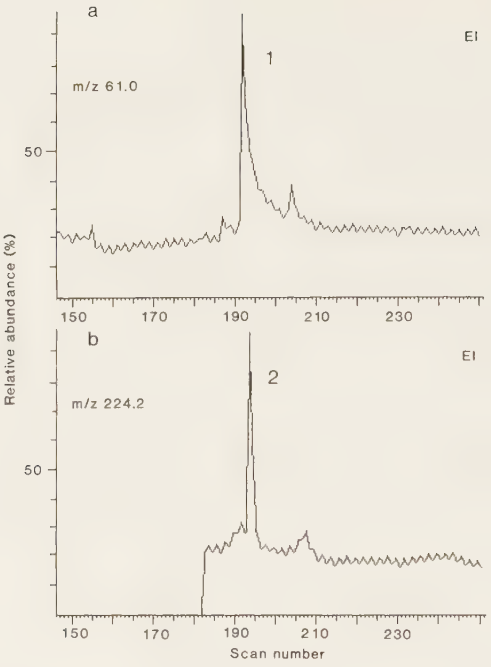


Fig. 6. Mass fragmentogram (single ion monitoring) of lipid extract of preputial gland secretion from one dominant male bank vole. Peaks (1) and (2) correspond to adducts of hexadecyl acetate fragments of (m/z 61.0) base peak from saturated acetates and of (m/z 224.2) M-60 resulting from the loss of acetic acid.

TABLE 1

Total amount of n-hexadecyl acetate in preputial glands of male bank voles. In the individuals examined one gland was extracted and its contents measured before treatment of the animal with testosterone. Ten days later the remaining gland was extracted and its contents extracted.

Individual numbers	Total amount (ng) n-hexadecyl acetate before treatment	after hormone treatment
1	11	46
2	21	93
3	5	47
4	42	47
5	24	359

DISCUSSION

The histological appearance of the preputial glands in male bank voles differed from that of rats (Montagna and Nobak 1946) and mice (Beaver 1960, Green 1966, Brown and Williams 1972) (Figs. 1b, 1d and 2d). The glands were not of the tubulo-alveolar type and the two secretory cell-types were not arranged in acini. Also, there was no obvious pattern of ducts. In many respects, the glands showed a great similarity to those of red-backed voles, described by Lindsay (1978). In both species granules and lipid droplets are produced by separate cell types. The most obvious difference between these two closely related species was that in bank voles cells producing lipid droplets were not arranged around the granule cells but the two cell types formed scattered groups in the gland. Furthermore, in the dominant male, lipid droplets were occasionally found in the granule cells. At the cellular level Beaver (1960) described granules and lipid droplets situated peripherally in the cells of rats. In the bank vole, however, both the granules and the lipid droplets were evenly scattered over the cytoplasm (Figs. 1c and 1d). Many odour-producing tissues are dependent upon androgens (see Adams 1980 for references). This is particularly important in controling glandular activity. It is well known that increased organ size normally is a clear sign of increased activity of the organ. In a study on male mountain voles (Microtus montanus), Jannet (1978) found a linear dosage response of the weight of the preputial glands to testosterone propionate treatment. In the present study the preputial glands of the testosterone treated bank voles were clearly hypertrophied, thus indicating an increased activity of the glands. This result confirms earlier findings (review by Brown and Williams, 1972) showing that androgens increase preputial gland size in rodents. Further support is the striking increase in the number of granules and lipid droplets in the treated specimens. Moreover, the histology of the preputial gland of the dominant male revealed a large amount of vesicles filled with secretion, and the hypertrophied cells were filled with granules. Lipid droplets, however, were found rather scarcely in this male (Fig. 2e). This may indicate that the lipid vacuoles act as reservoirs for the secretion before its release into the large vesicles prior to marking.

This in turn indicates that testosterone treatment increases the synthesis of odorous material or its biochemical precursers, but that it does not affect the release. Further stimuli, e.g. social interactions, are probably required for the release.

From gas chromatographic and mass spectral data, Spener et al. (1969) identified n-hexadecyl acetate in preputial gland secretion of male mice. Furthermore, from thin layer-, infrared layer- and gas chromatographic analyses they found indications for twelve more (mainly hexadecyl- and octadecyl-) acetates. In preputial gland secretion of bank voles, we could not discover octadecyl acetate (M=298.5) although we looked specificly for the (M+18) adduct ($CI-NH_3$). Testosterone treatment increases the synthesis of wax, alkyl acetates and alkyl glyceroles in male mice (Sansone-Bazzano et al. 1972). They showed that the lipid content varies quantitatively and qualitatively with both age and sex. A volatile fraction attractive to the opposite sex has been isolated from the preputial gland secretion in male rats (Gawienowski et al. 1975). Our results support the study by Nickerson et al. (1976), who showed that in female rats testosterone propionate induces a significant increase in the weight of the preputial gland. Stacewicz-Sapuntzakis and Gawienowski (1977) found indications of ethyl-, propyl-, isopropyl-, pentyl- and decyl acetates in preputial gland secretion of male rats. In our study the hexadecyl acetate values varied considerably (Table 1). An increase after the hormone treatment was, however, clear. In two specimens (3) and (5) the increase was particularly great. The histological picture of these specimens revealed a higher number of lipid droplets than in the other animals. Also the size of the droplets increased in these two specimens. The increased amount of both hexadecyl acetate and lipid droplets strongly suggests that these droplets contained hexadecyl acetate.

In species with dominance hierarchies, it is possible that the social status of individuals is identified by odours. Dominant and subordinate animals may be distinguished from differences in the amount of secretion and/or frequency of scent marking (Bronson 1976, Brown 1979, Stoddart 1980).

Our subordinate bank voles treated with testosterone did not change their behaviour towards dominant male type. The behaviour of a subordinate male may not necessarily change in relation to changes of androgen levels. Subordinate male mice remained so, also after implants of testosterone (Bronson 1976). This may be

due to the fact that subordination can be related to experience of defeats in agonistic interactions. Both urine marking (Bronson 1976, op.cit.) and release of substance from accessory glands may be under nervous control (Adams 1980). Thus, although marking substances which are normally found in dominant males, may be synthesized in subordinates treated with androgen, these substances may not be externally voided.

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